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(54) Title: METHODS OF DETECTING POLYNUCLEOTIDE ANALYTES

(57) Abstract

Sensitive and specific methods of detecting single–stranded polynucleotide analytes can be used in manual or automated diagnostic assays and to screen blood samples for the presence of infectious agents. The methods can be used to detect any single–stranded polynucleotide analyte whose sequence is known. Single–stranded DNA analytes which are present in a biological sample at a concentration of 0.1 fg/μl can be detected.

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METHODS OF DETECTING POLYNUCLEOTIDE ANALYTES

TECHNICAL AREA OF THE INVENTION

The invention relates to the area of detecting polynucleotide analytes. More particularly, the invention relates to diagnostic assays based on the detection of specific nucleotide sequences.

BACKGROUND OF THE INVENTION

The polymerase chain reaction (PCR) has had a substantial impact on the diagnosis of genetic and infectious disease. The ability of this method to amplify minute amounts of specific DNA sequences in a mixture of host DNA makes it a powerful diagnostic tool.

PCR, however, has inherent problems and limitations. The extreme sensitivity of PCR paradoxically leads to one of its major drawbacks, the occurrence of false positive results. Very small amounts of the amplified target sequence, of which up to 10^9 copies can be present in a single PCR solution, can contaminate laboratory equipment or reagents. The PCR product can even spread as airborne droplets in areas of sample or reagent preparation. This contaminating DNA can then serve as a template for further amplification, resulting in false positive results in subsequent samples. A common precaution against this type of error is the establishment of procedures to physically separate the steps of PCR reactions in a laboratory and/or the routine use of chemical and enzymatic methods for inactivating PCR products. These constraints add further cost and inconvenience to the use of PCR in routine laboratory environments and are not completely effective in preventing contamination.

Another problem with PCR-based diagnostic methods is that the clinical relevance of a positive PCR result is questionable when small numbers of pathogenic organisms are present in samples from persons who are clinically unaffected. Further, because PCR amplifies only a portion of the genome of an infectious agent, another source of error is the detection of nonviable organisms. In such instances, detection of cDNA by reverse-transcription PCR of messenger RNA encoded by the pathogenic organism could be misinterpreted as evidence of active infection. Finally, because PCR uses two primers to achieve exponential amplification, the method is sensitive to genetic variability of the target sequence.

Thus, there is a need in the art for reliable and convenient methods which can sensitively and specifically detect single-stranded DNA analytes in a biological sample.

SUMMARY OF THE INVENTION

It is an object of the invention to provide sensitive and specific methods for detecting the presence of a polynucleotide analyte in a biological sample. This and other objects of the invention are provided by one or more of the embodiments described below.

One embodiment of the invention is a method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample. A polynucleotide molecule on a solid support is detected. The polynucleotide molecule comprises a single-stranded polynucleotide analyte and one or more single-stranded polynucleotide probes which specifically hybridize to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded. At least one of the single-stranded polynucleotide probes is bound to the solid support. Detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

Another embodiment of the invention is a kit for detecting a single-stranded DNA analyte in a biological sample. The kit comprises at least one DNA probe which is single-stranded, a solid support, and a monoclonal antibody which

specifically binds to double-stranded DNA. The DNA probe comprises a first binding moiety. The solid support comprises a second binding moiety. The first and second binding moieties specifically bind to each other.

Yet another embodiment of the invention is a kit for detecting a single-stranded DNA analyte in a biological sample. The kit comprises a solid support and a monoclonal antibody which specifically binds to double-stranded DNA. The solid support comprises one or more single-stranded DNA probes.

Even another embodiment of the invention is a single-stranded DNA primer which consists of a sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 5, 8, 10, and 12.

The present invention thus provides the art with a method of detecting a single-stranded polynucleotide analyte in a biological sample. The method is sensitive, specific, and reliable, and can be used in manual or automated diagnostic or screening assays.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 demonstrates detection of CMV particles in serum using Amplification Independent DNA Assay (AIDA).

Figure 2 demonstrates the increase in sensitivity obtained after carrying out a polymerase elongation step after hybrid capture.

Figure 3 shows the results of an AIDA test on the sera of control and HIV-positive patients.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention solves problems inherent with exponential amplification methods by using a single polynucleotide probe to both capture a single-stranded polynucleotide analyte and to generate a detection signal. The method provides a sensitive and specific method of detecting a particular single-stranded polynucleotide analyte in a biological sample without need to resort to the elaborate and expensive procedures required with PCR-based methods. For example, the method is capable

of detecting a single-stranded DNA analyte present at a concentration as low as 0.1 fg/μl.

The single-stranded polynucleotide analyte can be DNA or RNA, preferably genomic or cDNA. If the presence of a DNA virus in a biological sample is suspected, specific sequences in the viral genome can be detected after extraction of genomic DNA from serum or whole blood (see Example 8, below). A single-stranded DNA analyte can be generated in the biological sample from a double-stranded starting material, for example, by heating or chemically treating the biological sample to denature double-stranded DNA. If the presence of an RNA virus is suspected, the RNA viral genome can be converted into single-stranded DNA using reverse transcriptase, as is known in the art (see Example 2, below). Similarly, the presence of any mRNA can be detected by first converting the mRNA into single-stranded DNA.

Methods of the invention can be used to detect the presence of pathogens, such as a bacteria, viruses such as hepatitis C (HCV), hepatitis B (HBV), hepatitis G (HGV), or human immunodeficiency virus (HIV), fungi, protozoa, parasites, or mycoplasma. Any organism which contains RNA or DNA can be detected. The method can be used to detect the presence of genetic mutations which have diagnostic or prognostic value. Pathogen contamination of food and drink supplies can also be detected using the method.

The biological samples used for diagnostic purposes can be, for example, samples such as tissue or cellular extracts, whole blood, serum, or plasma. Blood samples can be obtained by venepuncture or by accessing capillary veins of the finger or heel of a postnatal human. Tissue or cell samples can be obtained using appropriate biopsy methods. Prenatal samples of fetal blood or tissue can also be tested. Biological samples used for analytical purposes can be those described above or can be, for example, samples of food, drink, or bodies of water, such as ponds, rivers, lakes, or pools.

The presence of a single-stranded polynucleotide analyte in a biological sample is indicated by detecting a double-stranded polynucleotide molecule on a solid support. The double-stranded polynucleotide molecule is formed by specific hybridization of the single-stranded polynucleotide analyte with one or more single-

stranded polynucleotide probes. After hybridization, all or a portion of the single-stranded polynucleotide analyte can be double-stranded. At least one of the probes is bound to the solid support and thus is used both to capture the single-stranded polynucleotide analyte and to form a double-stranded polynucleotide portion which is then detected.

In one embodiment of the invention, AIDA (Amplification Independent DNA Assay), the presence of a single-stranded polynucleotide analyte, preferably a single-stranded DNA analyte, present in very low concentration in a biological sample can be detected independent of analyte amplification. Instead, the only amplification which may occur in AIDA is amplification of the number of regions of the single-stranded analyte which can be detected using a reagent which specifically binds to double-stranded polynucleotide molecules, such as an anti-double-stranded DNA antibody. This procedure is highly innovative because it eliminates problems related to analyte amplification, such as cost and contamination, and also provides high levels of sensitivity.

The AIDA assay can be carried out in any practical sample volume. Most conveniently, the AIDA assay is carried out in a 0.05 to 5 ml sample, preferably in a 0.1 to 1 ml sample. Typically, a biological sample is contacted with one or more polynucleotide probes, preferably DNA probes, which are single-stranded and complementary to one or more regions of the single-stranded DNA analyte. A DNA probe is complementary to a single-stranded DNA analyte if its nucleotide sequence will form hydrogen-bonded base pairs with a nucleotide sequence in the single-stranded DNA analyte. DNA probes which are complementary to any known nucleotide sequence can be synthesized chemically, using an automated oligonucleotide synthesizer, such as the PCR Mate - EP 391 DNA synthesizer (Applied Biosystems, Foster City, CA), using β-cyanoethyl phosphoramidite chemistry. This method is described in S.L. Beaucage, S.L. and M.H. Caruthers, *Tetrahedron Lett.*, 22, 1859-62 (1989). Additional quantities of the DNA probe can be generated, for example, using a DNA polymerase, as is known in the art.

The sensitivity of the method is influenced by the length of the single-stranded DNA probe. Suitable DNA probes are at least 20, 30, 40, 50, 60, 70, 80, 90, or 100

nucleotides in length, and DNA probes of at least 50 nucleotides are preferred. However, any probe, regardless of length or sequence, can be used.

Preferred DNA probes for use in the present invention are those which comprise the sequences shown in SEQ ID NOS:6, 9, 13, and 18. The nucleotide sequence shown in SEQ ID NO:6 is complementary to a portion of a hepatitis C genome. The nucleotide sequence shown in SEQ ID NO:9 is complementary to a portion of a hepatitis B genome. The nucleotide sequence shown in SEQ ID NO:13 is complementary to a portion of a hepatitis G genome. The nucleotide sequence shown in SEQ ID NO:18 is complementary to a portion of an HIV genome.

Preferably, the biological sample is contacted with the single-stranded polynucleotide probe in an aqueous solution. The solution can contain certain agents, such as polyvinylpyrrolidone, bovine serum albumin, the synthetic sucrose polymer "FICOLL 400," salmon sperm DNA, and yeast RNA, to decrease non-specific binding of the probe to proteins, polysaccharides, and nucleic acids. The step of contacting is carried out under conditions where single-stranded polynucleotide probes hybridize to complementary single-stranded polynucleotide analytes to form double-stranded polynucleotide molecules. Variables involved in determining hybridization conditions include probe length and concentration, concentration of single-stranded polynucleotide analyte, temperature, salt concentration, and the relative percentage of G-C versus A-T bonds which can be formed between the polynucleotide probe and the single-stranded polynucleotide analyte. Conditions suitable for hybridizing a particular probe-analyte pair can readily be determined by one of skill in the art. Basic manuals of recombinant DNA techniques, such as Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed. (Cold Spring Harbor Press, Cold Spring Harbor, New York), can be referred to for this purpose. Single-stranded polynucleotide molecules which do not specifically hybridize to the single-stranded polynucleotide probes employed can be eliminated, for example by one or more washing steps.

At least one of the polynucleotide probes which hybridize to the single-stranded polynucleotide analyte is used to affix the double-stranded polynucleotide molecules to a solid support. The single-stranded polynucleotide probe can be

attached to the solid support prior to or after hybridization with the single-stranded polynucleotide analyte, as is desired.

Any method known in the art can be used to attach the single-stranded probe to the solid support. For example, two binding moieties which specifically bind to each other can be used. The probe can comprise a first binding moiety, such as biotin. A biotin moiety can be bound to the 5' end of a DNA probe, for example, during automated synthesis by using Biotin-Phosphoramidite (Amersham), resulting in a biotinylated DNA probe. The solid support then comprises a second binding moiety, such as avidin or streptavidin. Alternatively, the first binding moiety can be avidin or streptavidin and the second moiety can be biotin. Other specific binding pairs, such as antibody-antigen pairs, can be used to bind the probe to the solid support. Any two binding partners can be used which specifically bind to each other with a K_D less than 10^{-7}, 10^{-8}, 10^{-9}, or preferably 10^{-12}.

Alternatively, the probe can be covalently bound to the solid support, for example by use of a covalent cross-linker such as 1-(*p*-azidosalicylamido)-4-(iodoacetamido)butane, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodimide hydrochloride, *N*-hydroxylsulfo-succinimidyl 4-azidobenzoate, sulfosucinimidyl 4-(*p*-azidophenyl)butyrate, sulfosuccinimidyl β-[alpha-methyl-alpha-(2-pyridyldithio)toluamido]hexanoate, 1,4-di-[3'-(d'-pyridyldithio) propionamido] butane, sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide) ethyl-1,3'-dithiopriopionate, or sulfo-succinimidyl 7-azido-4-methylcoumarin-3-acetate. The second binding moiety can be bound to the surface of the solid support by passive absorption or by use of a covalent cross-linker.

The solid support can be any surface to which the single-stranded probe can be attached. Suitable solid supports include, but are not limited to, glass or plastic slides, tissue culture plates, microtiter wells, tubes, or particles such as beads, including but not limited to latex, polystyrene, or glass beads. The efficiency of the method is increased if particles are used as the solid support. In this case, the particles can be placed in a tube for use as a fixed or removable cartridge or in a microtiter well, for use in either manual or automated assays.

Detection of the presence of a double-stranded polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample. A variety of methods are available to detect the presence of double-stranded polynucleotide molecules on the solid support. Preferably, a reagent which specifically binds to double-stranded polynucleotide molecules, particularly double-stranded DNA molecules, can be employed for this purpose. Such reagents include, but are not limited to, proteins (such as antibodies, antibody fragments, and DNA binding proteins) and dyes specific for double-stranded DNA, such as ethidium bromide. Preferably, the reagent is an antibody, such as a polyclonal or monoclonal antibody, or an antibody fragment, such as an Fab, $F(ab)'_2$, or a single-chain antibody. Most preferably, the antibody has an affinity for double-stranded DNA molecules at least 100-, 500-, 1,000- or 10,000-fold greater than for single-stranded DNA molecules. A monoclonal antibody with these properties can be produced, for example, by conventional procedures for generating antibody-secreting hybridomas, using spleen cells from an animal which is susceptible to autoimmune disease, such as an MRL/lpr mouse.

The bound antibody can be directly or indirectly coupled to a reporter system, in which a detectable signal is generated which is proportional to the amount of double-stranded DNA which is bound to the solid support. For example, the antibody can comprise a detectable label, such as a radioactive, fluorescent, chemiluminescent, or colloidal gold label. Methods for detecting these labels, such as spectrophotometry, autoradiography, nephelometry, fluorimetry, or flow cytometry, are well known in the art. Alternatively, the antibody can be detected using an indirect immunochemical method, for example, by adding peroxidase-or alkaline phosphatase-labeled protein A after binding the double-stranded DNA antibody and carrying out a chromogenic reaction, or by use of a second antibody which is labeled and immunoreactive with the first antibody. Optionally, the presence of antibody bound to double-stranded DNA molecules can be detected by monitoring changes in electrical conductance of a solution in contact with stranded DNA molecules, as is known in the art.

Preferably, the antibody and detection system used are capable of detecting double-stranded DNA molecules which are present in the biological sample at a concentration of less than 0.3 pg/μl. Most preferably, double-stranded DNA molecules which are present in the biological sample at a concentration of less than 5 fg/μl, less than 1 fg/μl, less than 0.5 fg/μl, or less than 0.1 fg/μl can be detected. A preferred detection system is the ETI-2 system, available from DiaSorin and described in Example 1, below.

Ideally, the method of detecting the amount of antibody bound to double-stranded DNA molecules provides a signal which is at least 2-, 5-, 10-, 20-, or 50-fold higher than a background signal. The sensitivity of the detection step can be increased, for example, by providing additional binding epitopes for the anti-double-stranded DNA antibody. Additional binding epitopes can be provided in the hybridization step, by adding additional single-stranded DNA probes which are complementary to the single-stranded DNA analyte but whose nucleotide sequence does not overlap that of the DNA probe used to affix the analyte to the solid support.

Additional binding epitopes can also be provided by carrying out a polymerase elongation step, either before or after the analyte is affixed to the solid support, before the addition of the antibody. In this way, single stranded portions of the DNA analyte can be converted to double-stranded DNA, creating a considerable increase in the number of epitopes which can be recognized by the anti-double stranded DNA antibody. Because this reaction is carried out at 37° C for 10 minutes, any conventional, non-thermostable polymerase, such as T4, T7, or Klenow, can be used. It is also possible to provide additional binding epitopes by coupling additional double-stranded DNA, either in linear form or as dendrimers, to the single-stranded DNA probe.

Detection of double-stranded polynucleotide molecules can be either qualitative or quantitative. Qualification can be accomplished, for example, by comparing the amount of detectable label on an antibody which specifically binds to a double-stranded DNA with a standard curve or by providing an internal standard against which to measure the amount of antibody which is bound to double-stranded DNA in the biological sample.

The AIDA method provides a unique tool for simultaneously detecting the presence of two or more different single-stranded polynucleotide analytes in a single sample using a single reaction. This ability is useful, for example, to screen erythrocyte concentrates prepared from donated blood for the presence of HIV, hepatitis B, hepatitis G, and hepatitis C viral sequences, in order to avoid contamination which can occur during the window period. In this case, the biological sample is contacted with a mixture comprising single-stranded DNA probes specific for each single-stranded DNA analyte to be detected. If desired, a biological sample which provides a positive detection signal when screened can then be analyzed with individual probes to identify the contaminating single-stranded DNA analyte.

In another embodiment of the invention, additional copies of a single-stranded DNA analyte are synthesized to enrich the biological sample for the single-stranded DNA analyte. The size of the biological sample can thus be smaller than the biological sample used in the AIDA method above. This method is termed LEDIA (Linearly Enriched DNA Immunoassay). Typically, the LEDIA method can be performed using a biological sample of 100 µl.

Additional copies of the single-stranded DNA analyte can be synthesized, for example, using a thermostable DNA polymerase isolated from an organism such as *Pyrococcus furiosus* or *Thermus aquaticus*. Any commercially available thermal cycler can be used.

In the present invention, occurrence of exponential amplification after a sufficient number of cycles is an undesired result which can be avoided by careful choice of enrichment conditions. The efficiency of linear versus exponential enrichment of a target sequence depends on several parameters, including number of cycles, sequence and concentration of primer, temperature, and amount of DNA polymerase. Optimization of these variables is discussed in detail in Example 12, below.

Preferably, the step of synthesizing employs one single-stranded DNA primer so that additional copies of the complement of the single-stranded DNA analyte are not synthesized. Most preferably, the kinetics of enrichment of the biological sample for the single-stranded DNA analyte are linear. To achieve linear enrichment for the

single-stranded DNA analyte, fewer than 50 polymerization cycles are employed. Most preferably, fewer than 49, 48, 47,46, 45, 40, 39, 38, or 30 polymerization cycles are employed.

Primers for use in the LEDIA method must be specifically identified by routine testing in a LEDIA assay. Testing of several possible primers generally results in identification of a primer which is suitable for enriching a biological sample for a particular single-stranded DNA analyte. Examples of single-stranded primers which are suitable for use in the LEDIA method are primers which comprise one of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 4, 5, 8, or 12. Primers with the nucleotide sequences shown in SEQ ID NOS:1-5 can be used to enrich a biological sample for portions of a hepatitis C genome. Primers with the nucleotide sequence shown in SEQ ID NO:8 can be used to enrich a biological sample for a portion of a hepatitis B genome. Primers with the nucleotide sequence shown in SEQ ID NO:12 can be used to enrich a biological sample for a portion of a hepatitis G genome.

Detection of a single-stranded DNA analyte using the LEDIA method does not require any chemical modification of the analyte. Furthermore, the LEDIA method avoids the risk of contamination which is inherent in exponential amplification techniques such as PCR. Because the enriched strand contains no sequences which are complementary to the DNA probe, the enriched strand cannot serve as a template for further reactions. The risk of contamination is therefore minimized. The LEDIA method therefore can sensitively and specifically detect a single-stranded DNA analyte without the occurrence of false positives which frequently occur with exponential amplification techniques. This feature of the LEDIA method avoids the need to set up cumbersome and often expensive procedures for physically isolating different steps of the reaction, such as treatment of samples, preparation of reagents, execution of the reaction, and analysis of the end-products of the reaction.

A further advantage of the single-primer-based LEDIA method is that it is less sensitive to genetic variability of the single-stranded DNA analyte than methods

based on the use of two primers, such as PCR. This feature of LEDIA is particularly relevant for detection of viruses which have high genetic variability, such as HIV.

The LEDIA method is also particularly well-suited for quantitative applications. Because the enrichment of the biological sample for the single-stranded DNA analyte is linear rather than exponential, results can be reliably compared, for example, to a standard curve.

As with the AIDA method described above, the LEDIA method can be used to detect two or more different single-stranded DNA analytes in the same biological sample. Two or more different single-stranded DNA primers can be used to enrich the biological sample for two or more single-stranded DNA analytes. For example, primers which can be used for linear enrichment of portions of hepatitis B, hepatitis C, hepatitis G, and HIV genomes can be used to amplify each of these analytes in one biological sample.

The invention also provides kits which can be used for diagnosis or to screen a biological sample for the presence of one or more polynucleotide analytes. Kits which comprise one or more single-stranded DNA probes, a solid support, and a monoclonal antibody can be used to detect a single-stranded DNA analyte in a biological sample. The DNA probe can comprises a first binding moiety and the solid support can comprises a second binding moiety which specifically binds to the first binding moiety, as described above. Alternatively, the solid support can comprise one or more single-stranded DNA probes.

Kits can additionally comprise written instructions for detecting a single-stranded DNA analyte using the AIDA method. Optionally, a thermostable DNA polymerase, such as a polymerase isolated from *Thermus aquaticus* or *Pyrococcus furiosus*, and a primer for linear enrichment of a single-stranded DNA analyte can be included in a kit, together with written instructions for detecting the single-stranded polynucleotide using the LEDIA method. Kits which can be used to detect two or more distinct single-stranded DNA analytes in a single biological sample can be provided by including additional DNA probes and single-stranded DNA primers.

The AIDA and LEDIA methods can also be employed in partially or fully automated assays. An automated microwell plate reader can be used to carry out the

detection step of the method. Alternatively, an automated plate processor can be used to carry out the entire method. Optionally, solid supports such as beads cn be placed in a tube or cartridge, as described above.

The following are provided for exemplification purposes only and are not intended to limit the scope of the invention which has been described in broad terms above.

EXAMPLE 1

Example 1 demonstrates detection of CMV-DNA in sera by AIDA.

Extraction of DNA. CMV particles (TOWNE strain, AD 169) were obtained from ABI, Rivers Park, Guilford Road, Columbia, MD 21048. The viral particle count was 3.85 billion/ml. CMV particles were serially diluted in 200 µl of normal serum obtained from a CMV negative blood donor. DNA was extracted from the spiked serum using a QIAamp Blood kit by Qiagen (Cat. No. 29104) according to the manufacture's instructions. Qiagen protease (25 µl) was added to a 1.5 ml centrifuge tube with 150 µl of serum, and the volume was adjusted to 200 µl with PBS. Two hundred microliters of buffer AL was added to the tube. The contents were vortexed for 15 seconds and incubated at 70°C for 10 minutes. After mixing, 210 µl of ethanol (96-100%) was added, and the sample was mixed again by vortexing.

The mixture was applied carefully to a QIAamp spin column and centrifuged at 6000 x g (8000 rpm) for 1 minute. The column was placed in a clean 2 ml collection tube, and the collection tube containing the filtrate was discarded. The QIAamp spin column was carefully opened, and 500 µl of Buffer AW was added. The spin column was centrifuged at 6000 x g (8000 rpm) for 1 minute. The column was placed in a clean 2 ml collection tube, and the collection tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and another 500 µl of Buffer AW was added. The column was again centrifuged at full speed for 3 minutes. After centrifugation, the column was placed in a clean 1.5 ml microfuge tube, and the collection tube containing the filtrate was discarded.

Hybridization. The DNA was eluted from the column with 50 µl of buffer

AE preheated to 70°C. The samples were incubated at 70°C for 5 minutes in a heating block and then centrifuged at 9000 rpm for 1 minute. The tube was rotated in the centrifuge and centrifuged again for another minute.

Fifteen microliters of 1N NaOH was added to 50 µl of the sample and mixed by vortexing. The sample was boiled for 5 minutes at 100°C, placed on ice, and centrifuged briefly. Ten microliters of a biotinylated, CMVS3-12-specific probe (diluted 1 ng/µl in H_2O) was added to the sample. The sample was boiled for 5 minutes and mixed by vortexing. Following the addition of 22.5 µl of 666 mM HCl and 333 mM Tris pH 8, 14.3 µl of hybridization buffer (5.5 µl of 20 x SSC, 4.4 µl of 50X Denhard's solution, 4.4 µl of 500 mM EDTA, pH 8) was added. The sample was mixed by vortexing and centrifuged briefly.

Detection with anti-double-stranded DNA antibody. After a one-hour incubation at 50°/60°C with shaking, the tube was placed on ice, then centrifuged briefly. The whole volume was dispensed in one microwell of a GEN-ETI-K DEIA coated strip (DiaSorin, code number M2600998143). The strip was covered with a cardboard sealer and incubated overnight at 4°C.

The strip was washed 5 times with wash buffer ETI-2 (DiaSorin) using an ETY-SYSTEM washer (DiaSorin) or its equivalent. Anti-double-stranded DNA antibody 27-14-D9 was diluted 1:50 in anti-double-stranded DNA diluent (DiaSorin, code number M2600999839), and 100 µl of the diluted antibody was added to the well. After a 30 minute incubation at 37°C, the well was washed 5 times with ETI-2 buffer. The sample was then incubated for 30 minutes at 37°C with 100 µl of protein A BS3 enzyme tracer (DiaSorin, code number M2600999876) diluted 1:50 in tracer diluent (DiaSorin, code number M2600999840), and washed 5 times with ET1-2 buffer. The sample was incubated for 30 minutes at room temperature in the dark with 100 µl of a 1:1 mixture of ETI-2 chromogen (DiaSorin, code number M260099803) and ETI-2 substrate (Diasorin, code number M260099804). DiaSorin M26000654 blocking reagent (200 µl) was added, and the absorbance of the specimen was measured with a spectrophotometer at 450-630 nm.

At least 100 particles of CMV could be detected, as shown in Figure 1.

EXAMPLE 2

This example demonstrates detection of hepatitis C sequences in serum samples using AIDA with the following steps: (a) extraction of total RNA from a serum pellet, (b) conversion of RNA into first strand cDNA using the enzyme reverse transcriptase, (c) liquid-phase hybridization of cDNA with a biotinylated probe, and (d) capture of cDNA-probe hybrids onto microplate wells coated with streptavidin, and (e) detection of the specific hybrids by a DNA ELISA, using the anti-double-stranded DNA monoclonal antibody 27-14-D9. Each step of the method was carried out as follows.

(a) Extraction of total RNA from serum. A 1.5 ml serum sample was centrifuged at 14,000 x g for one hour to pellet viral particles. The supernatant was separated from the pellet and conserved. The pellet was resuspended in 100 μl of supernatant and processed for RNA extraction. Extraction of the HCV RNA genome was accomplished using the Tripure Isolation Reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. The final pellet containing RNA was resuspended in 30μl of diethyl-pyrocarbonate-treated H_20 (SIGMA Chemicals, St. Louis, MO).

(b) Conversion of RNA into first strand cDNA by the enzyme reverse transcriptase. First strand cDNA synthesis was performed using the reverse transcriptase enzyme Superscript II (Life Technology), using random examers (Life Technology) as cDNA primers. The RNA sample (11 μl) and 1 μl of examers (250 ng/μl) was heated at 70°C for 10 minutes and cooled at room temperature for 10 minutes. To each tube was added 4 μl of 5x first strand synthesis buffer (250 mM Tris HCl , pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 μl of 0.2 M DTT, 1 μl of dNTPs (10 mM each), and 1μl of Superscript, in a total reaction volume of 20 μl. The mixture was incubated at 42°C for 1 hour. After that time, the enzyme was inactivated by heating the sample to 65°C for 10 minutes.

(c) Liquid-phase hybridization. Complementary DNA obtained from viral RNA and 10 ng of a biotinylated HCV probe having the nucleotide sequence shown in SEQ ID NO:6 (1 ng/ml) were mixed, heat-denatured at 100°C for ten minutes, quickly cooled on an ice bed, then hybridized for 90 minutes at 65°C in a

hybridization mixture containing 1 x SSC (150 mM NaCl, 15 mM trisodium citrate), 2x Denhardt's solution (1x Denhardt's solution is 0.2 g/l bovine serum albumin, 0.2 g/l "FICOLL," and 0.2 g/ polyvinylpirrolidone), 10 ng of the biotinylated HCV probe having the nucleotide sequence shown in SEQ ID NO:6, and the entire cDNA reaction (20 μl), in a total volume of 100 μl. At the end of the incubation, the hybridization mixture was quickly cooled in dry-ice-ethanol bath (or, alternatively, in liquid nitrogen bath) for five seconds, and then maintained on an ice bed for five minutes.

(c) Hybrid capture onto streptavidin-coated microtiter wells. The cooled hybridization mixture was added to streptavidin-coated microtiter wells and incubated overnight at 4°C.

(d) Detection of the specific hybrids by DNA ELISA with anti-double-stranded DNA antibody. The captured HCV sequence was detected by a procedure involving an anti-double-stranded DNA antibody able to discriminate single-stranded from double-stranded DNA. The double-stranded DNA hybrids were visualized by generating a colorimetric signal.

The assay was carried out as follows. After capture of double-stranded hybrids, the wells were washed seven times with 300 μl of washing buffer (6.7 mM phosphate buffer, pH 6.4, 0.13 M NaCl, 0.1% of the detergent "TWEEN 20"). Anti-double stranded DNA antibody 27-14-D9 was added and allowed to react for 30 minutes at 37°C, then excess antibody was removed by another seven washes in washing buffer.

Double-stranded DNA-antibody complexes were detected by adding to the wells a solution containing a horseradish peroxidase-labeled goat-antimouse Ig antibody, incubating for 30 minutes and, after an additional five washes, adding a chromogenic solution (27 g/l tetramethylbenzidine and 0.1 ml/l hydrogen peroxide). After 30 minutes the chromogenic reaction was stopped with 0.2 ml of 1N H_2SO_4, and the optical densities of the samples were read at 450 nm on an automatic microtiter spectrophotometer (ETI-System Fast Reader, Sorin Biomedica, Saluggia, Italy).

Five HCV-positive samples and five HCV-negative samples were analyzed by AIDA in two separate experiments, as shown in Table 1.

Table 1. Detection of HCV sequences by AIDA in serum samples

sample	OD 450 nm
HCV+ 1	0.466
HCV+ 2	0.421
HCV- 1	0.136
HCV- 2	0.141
HCV+ 4	0.617
HCV+ 6	0.649
HCV+ 10	0.509
HCV- A	0.372
HCV- B	0.386
HCV- C	0.404

These results demonstrate that viral sequences from HCV can be detected in serum sample using AIDA after enzymatic conversion of viral RNA into cDNA, without the need for amplification or enrichment of the viral sequences.

EXAMPLE 3

This example demonstrates the use of the AIDA method to detect HIV sequences in the serum of HIV-infected patients.

Plasma from HIV-infected patients was collected in EDTA or in sodium citrate; heparinized plasma can also be used. RNA was isolated from the serum samples using a QIAamp VIRAL RNA kit by QIAGEN. The precipitate was redissolved in buffer AVL/carrier RNA by incubation at 80°C for not more than 5 minutes. Prepared buffer AVL (560 µl) was added to a 1.5 ml centrifuge tube. Plasma (140 µl) was added to the tube and the mixture was vortexed and incubated at room temperature 10 minutes. The sample was mixed thoroughly with 560 µl of

ethanol.

Carefully, 630 µl of the ethanol-sample mixture was applied to the QIAamp spin column and centrifuged at 6000 x g (8000 rpm) for 1 minute. The column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. The spin column step was repeated, then the QIAamp spin column was carefully opened and 500 µl of buffer AW was added. The column was centrifuged for 1 minute and placed in a clean 2 ml collection tube. The tube containing the filtrate was discarded. Another 500 µl of buffer AW was added to the spin column, and the column was centrifuged at 14,000 rpm (20,000 x g) for 3 minutes. The spin column was placed in a clean 1.5 ml centrifuge tube, and the tube containing the filtrate was discarded.

RNA was eluted from the column with 50 µl of preheated (80°C) RNase-free water incubated at 80°C for 5 minutes in a heating block, and centrifuged at 9000 rpm for 1 minute. The tube was rotated 180° in the centrifuge and spun again for another minute.

cDNA was synthesized using the SuperScript TM II RNase H-reverse transcriptase enzyme (Gibco BRL Cat. No. 18064-014). Two microliters of an anti-sense GAG specific primer (250 mg/µl) was added to 50 µl of RNA in Rnase free water. The anti-sense GAG specific primer had the following sequence: 5'CTATGTGCCCTTCTTGCCACAAT-3' (SEQ ID NO:19). This primer matches the sequence of HIV-1 B perfectly and has not more than 3 mismatches with the other subtypes and 5 mismatches with HIV subtype O. These mismatches should not influence the synthesis of the specific cDNAs, however, we have also verified that for this AIDA application it is possible to utilize random primers that eliminate the eventual problems linked to the presence of different subtypes and quasi species.

The RNA and primer were incubated for 5 minutes at 70°C and for 10 minutes at room temperature. After a brief centrifugation, 28.5 µl of the following mix was added to each sample: 16 µl of 5 X first strand buffer (provided with the enzyme), 8 µl of 0.1 M DTT (provided with the enzyme), 4 µl of 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP and dTTP at neutral pH, and 0.5 µl of SuperScript reverse transcriptase (200 U/µl). The mixture was then incubated for 60

minutes at 42°C.

Eight microliters of 1N NaOH in and the 80 µl of first strand cDNA were added to a 0.5 ml centrifuge tube, incubated for 5 minutes at 100°C, placed on ice for 2 minutes, and centrifuged briefly. After centrifugation, the tube was placed on ice and 8 µl of 1N HCl and 13.9 µl of hybridization buffer (5 µl of 20X SSC, 4 µl of 50X Denhard's solution, 4 µl of 0.5 M EDTA, pH 8, and 0.9 µl of 50X TE) were added five microliters of a gag-specific biotinylated sense probe (2 ng/µl diluted in 1X TE buffer) was added, and the mixture was preheated at 70°C for 5 minutes. The sequence of the probe is 5'biotGGGATTAAATAAAATAGTAAGAATGTATAGCCCTACCAGCA-3' (SEQ ID NO:20).

Following a 45 minute incubation at 60°C, the tube was place on ice and centrifuged briefly. The whole volume was dispensed into one streptavidin coated well and incubated overnight at 4°C.

The well was washed 6 times with wash buffer ETI-2 using an ETI-SYSTEM washer or the equivalent. After a 30 minute incubation at 37°C, the well was washed again 6 times and 100 µl of anti-double-stranded DNA antibody diluted 1:20 in anti-double-stranded DNA diluent was added. The well was incubated for 30 minutes at 37°C. The well was again washed 6 times, and 100 µl of chromogen/substrate (1:1) was added. After a 30 min incubation at room temperature in the dark, 200 µl of blocking reagent was added and the absorbance of specimen was immediately measured with a spectrophotometer at 450 nm.

This method accurately detected HIV sequences in four infected patients with high viremia and did not detect HIV sequences in two non-infected blood donors.

EXAMPLE 4

This example demonstrates that the sensitivity of AIDA can be further improved by adding mononucleotides and a nucleic acid polymerase capable of producing an extension product from the captured target DNA or cDNA/primer complex. This extension product creates new double-stranded DNA that, in turn,

provides new target epitopes for recognition by the anti-double-stranded DNA antibody.

Different dilutions of HIV stock solution were transcribed in duplicate into cDNA as described above and hybridized with a biotinylated gag probe (SEQ ID NO:20). The two groups of hybridization mixtures were added to separate streptavidin-coated microtiter wells and incubated overnight at 4°C.

After capture of hybrids, one plate was washed five times with 300 µl of washing buffer, and each well received 0.2 units of T4 polymerase and 80 µM of each of the four dNTPs. The other microtiter plate, which contained the same hybridization mixtures, was left untreated. The plates was incubated for 30 minutes at 37°C. Both plates were washed 5 times with washing buffer, and anti-double-stranded DNA antibody was added and allowed to react for 30 minutes at 37°C. Detection of bound anti-double-stranded DNA antibody was accomplished by adding to the wells a solution containing Protein A labeled with horseradish peroxidase for 30 minutes at 37°C. After five washes, a chromogenic solution was added. The chromogenic reaction was stopped with 0.2 ml of 1N H_2SO_4, and the optical densities were read at 450 nm using an automatic microtiter spectrophotometer.

The results shown in Figure 2 clearly show that the addition of the polymerase elongation step after the hybrid capture increased the sensitivity of the assay by at least two log units. Figure 3 shows the results of an AIDA test on the sera of nine blood donors (D1-D9) and with the sera of 9 HIV positive patients. The AIDA test accurately detected HIV sequences in eight of the nine HIV positive patients.

EXAMPLE 5

Using the method described below we analyzed panels of HIV viremic sera and compared the results with those obtained using reference methods of Roche and Chiron.

RNA was extracted from HIV viremic sera obtained from the Mayo Clinic using a QIAamp VIRAL RNA kit by QIAGEN, as described in Example 3. cDNA synthesis, hybridization, and detection were carried out as described in Example 3,

except that after the first washings of the microwell, 100 µl of a solution containing 50 mM Tri, pH 8.8, 15 mM $(NH_4)_2SO_4$, 7 mM $MgCl_2$, 01 mM EDTA, pH 8, 10 mM β-mercaptoethanol, 0.02 mg/ml BSA, pH 8.8, 80 µM NTPs, and 0.2 units of T4 DNA Polymerase (Boehringer Mannheim code 1004794) were added to the well, incubated for 30 minutes at 37°C and washed 6 times before addition of the anti-double-stranded DNA antibody.

The results are shown in Table 2 and demonstrate that, even using a relatively insensitive colorimetric detection method the AIDA method could easily detect the presence of 5000 virions in a sample.

Table 2

SENSITIVITY AND SPECIFICITY OF FIRST GENERATION HIV-AIDA

#	ROCHE	CHIRON	AIDA O.D. 450	AIDA RESULTS
1	742685	ND	2,068	+
2	102031	6608	0,600	+
3	479523	18740	0,210	+
4	264596	60580	1,800	+
6	105061	28090	0,520	+
7	159704	27190	1,260	+
8	216667	24310	0,600	+
15	63543	3891	0,290	+
16	198	<500	0,108	-
18	<188	<500	0,110	-
20	<223	<500	0,210	-
21	403	<500	0,100	-
22	<235	<500	0,104	-
23	395	<500	0,180	-
24	<247	<500	0,112	-
5	<259	<500	0,250	+
9	423	<500	0,146	-
10	333	<500	0,390	+
11	497	<500	0,115	-
12	<197	<500	0,130	-
13	212	<500	0,140	-
14	348	<500	0,201	-
D			0,119	-
D			0,146	-

#	ROCHE	CHIRON	AIDA O.D. 450	AIDA RESULTS
17	720690	102100	1,850	+
19	43180	4592	0,540	+
25	98726	6333	0,720	+
37	112225	10260	0,480	+
38	818529	111700	0,720	+
41	294600	18960	0,360	+
30	131243	7390	0,460	+
34	10770	1756	0,173	-
31	770	<500	0,093	-
32	394	<500	0,089	-
33	334	<500	0,120	-
35	345	<500	0,425	+
36	360	<500	1,135	+
39	572	<500	0,120	-
40	175	<500	0,132	-
42	<222	<500	0,130	-
26	341	<500	0,096	-
27	<336	<500	0,131	-
28	<250	<500	0,098	-
29	<146	<500	0,080	-
D			0,126	-
D			0,120	-

EXAMPLE 6

This example compares detection of HIV polynucleotide sequences in sera of HIV-infected patients using the AIDA method and T4 elongation step and using PCR in combination with the DEIA method (see Mantero *et al.*, Clin. Chem. 37, 422-29, 1991). RNA was extracted from sera using a QIAmp viral RNA kit by Qiagen (cat No. 295904) and reverse transcribed into cDNA using SuperScript II reverse transcriptase, as described above.

Hybridization was carried out as follows. Eighty microliters of cDNA (total cDNA reaction) and 8 µl of 1N NaOH were added to a 0.5 ml tube and mixed carefully by pipetting. The tube was incubated for 5 minutes at 100°C, chilled on ice for 5 minutes, then spun in a minicentrifuge. A pre-mixed solution containing 8 ml of HCl and 4 ml of 1M Tris, pH 7.5, was mixed carefully by pipetting. Sixteen microliters of hybridization buffer (6 µl of 20X SSC, 4.8 µl of 0.5M EDTA, pH 8.0, 4.8 µl of 50X Denhardt's solution, 0.9 µL of 50X TE buffer) was added to each tube. Then, 5 µl (5 ng) of HCV specific 5' biotinylated probe (CORE-890), pre-warmed at 75°C for 10 minutes, was added to each tube. The probe spans an HCV sequence in the region coding for the core protein. This probe sequence is conserved among HCV subtypes. The sequence of the probe is:
5' GGT CAG ATC GTT GGT GGA GTT TAC TTG TTG CCG CGC AGG G 3'
(SEQ ID NO:21).

The mixture was incubated at 50°C for one hour, chilled on ice for 5 minutes, and spun. Reactions (120 µl) were dispensed into the microwells of a streptavidin-coated microtiter plate (GEN-ETI-K DEIA), which was prechilled on an ice bed. The plate was covered with a cardboard sealer and incubated overnight at 4°C. The wells were washed 6 times with wash buffer ETI-2 using an ETI-System washer or the equivalent, and DNA was elongated using T4 polymerase, as described in Example 3. Double-stranded DNA was detected using anti-double-stranded DNA antibody, as described above.

Using the above described method we first tested sera from 7 HCV positive patients and of two blood donors. Sera were tested using AIDA and using PCR and DEIA as a reference method.

Table 3 shows that there was a complete concordance between the data obtaining using PCR and using AIDA.

TABLE 3. Detection of HIV sequences in sera of HIV-infected patients.

Sample	PCR (gel)	DEIA (OD)	AIDA (OD)
1	-	1.145	2.380
2	-	0.897	2.593
3	-	0.611	1.337
4	+	>3.000	2.610
5	+	2.470	1.819
6	+	>3.000	2.308
7	+	>3.000	>3.000
8*	-	0.054	0.063
9*	-	0.047	0.062

*healthy blood donors

In order to compare the sensitivity of AIDA with PCR, we tested different dilutions of cDNA obtained from the serum of an HCV positive patient with each of the two methods. Table 4 shows that the sensitivity of AIDA is comparable to the sensitivity of PCR.

TABLE 4. Dilution Test: Comparison of PCR/DEIA and AIDA

HCV positive serum pool	PCR gel	PCR +DEIA*	AIDA*
Dilution			
1:1	+	2.868	>3.000
1:5	+/-	0.989	2.258
1:10	-	0.430	1.390
1:20	-	0.089	0.760
1:40	-	0.066	0.207
1:80	-	0.081	0.154
1:160	-	0.062	0.115
1:320	-	0.107	0.138
1:640	-	0.052	0.134
positive serum (diluent)	-	0.069	0.190
positive control (HCV + pool)	+	>3.000	>2.846
healthy blood donor	-	0.069	0.169

*values in optical densities, 450 nm

EXAMPLE 7

This example demonstrates detection of HIV sequences in serum samples using AIDA.

The detection of HIV sequences in serum samples by AIDA involved exactly the same procedures as for HCV, except for the use of a different specific biotinylated probe (JA-Pol). This probe has the nucleotide sequence shown in SEQ ID NO:18. Four HIV-positive samples and one HIV-negative sample were analyzed by AIDA, as shown in Table 5.

Table 5. Detection of HIV sequences by AIDA in serum samples

sample		OD 450 nm
HIV+	FRRI	0.624
	MOCO	1.008
	EMBO	0.618
	RIVE	0.230
HIV-	DS	0.173

These results demonstrate that viral sequences from HIV can be detected in serum samples by AIDA after enzymatic conversion of viral RNA into cDNA, without the need for amplification or enrichment of the HIV sequences.

EXAMPLE 8

This example demonstrates the detection of human genomic sequences encoding the chemokine receptor CC-CKR5.

For this assay, genomic DNA was extracted from whole blood, and the DNA sample was processed for hybridization with the same procedures described above for cDNA hybridization, except for the use of the specific biotinylated probe, Fl2, having the nucleotide sequence shown in SEQ ID NO:17.

Extraction of genomic DNA from whole blood. Two hundred microliters of whole blood was diluted to 1 ml with buffer A (20 mM Tris HCl, pH 8.00, 5 mM EDTA), then centrifuged at 4000 x g for 15 minutes at 4°C. The supernatant was decanted, and the pellet was washed twice in buffer A, resuspended in 1 ml of the same buffer containing 0.4% SDS and 100 μg/ml proteinase K, and digested overnight at 37°C. At the end of the incubation, one-third volume of 5 M NaCl was added, mixed by inversion, and centrifuged at 3500 x g at 4°C for 15 minutes. One volume of cold absolute ethanol was added to the supernatant and mixed by

inversion. The DNA precipitate was captured on the tip of a flamed Pasteur pipette. The DNA was resuspended to a final volume of 1 ml in 10 mM Tris HCl, pH 8.00, 1 mM EDTA buffer (TE buffer).

Serial two-fold dilutions of DNA were evaluated by AIDA for detecting CC-CKR5 sequences, as shown in Table 6.

Table 6. Detection of CC-CKR5 sequences by AIDA in human genomic DNA samples

DNA, μg	OD 450 nm
2.5	2.093
1.25	1.885
0.6	1.216
0.3	0.816
0.15	0.700
0	0.232

These results demonstrated that AIDA can detect a specific genomic DNA sequence embedded in a complex DNA mixture in a specific and sensitive manner, without any amplification or enrichment of the specific sequence itself.

EXAMPLE 9

This example demonstrates detection of hepatitis C virus (HCV) in serum samples using the LEDIA method.

As HCV is a positive-strand RNA virus, its RNA genome must be preliminarily extracted from a biological sample such as serum and converted into DNA before any process involving a DNA-dependent DNA polymerase can be performed. Thus, linear enrichment of the HCV genome for detection involves the following steps: (a) extraction of total RNA from serum, (b) conversion of RNA into first strand cDNA by the enzyme reverse transcriptase, (c) specific linear enrichment of cDNA sequences derived from the target RNA, and (d) detection of the enriched single-stranded HCV sequence.

(a) Extraction of total RNA from serum. The extraction of HCV RNA genome from 100 μl of serum was carried out using the Tripure Isolation Reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. The final pellet containing RNA was resuspended in 30μl of diethyl-pyrocarbonate-treated H_20 (SIGMA Chemicals, St. Louis, MO).

(b) Conversion of RNA into first strand cDNA by the enzyme reverse transcriptase. First strand cDNA synthesis was performed using the reverse transcriptase enzyme Superscript II (Life Technology) using random examers (Life Technology) as cDNA primers, as described in Example 1, above.

(c) Specific linear enrichment of cDNA sequences derived from the target RNA. A reaction mixture of 100 μl was prepared, containing 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, $MgCl_2$ 2 mM, 16 mM $NH_4(SO_4)_2$, 67 mM Tris Cl, pH 8.8, 100 pmoles of a single HCV primer (either HCV A, B, G or 2CH,), 50 units/m of AmpliTaq (Perkin Elmer-Cetus, Norwalk, CT). To this mixture was added 1 μl of cDNA derived either from HCV positive patients or healthy blood donors negative for any HCV marker. This mixture was subjected to 35 cycles, each composed of three steps: denaturation at 94°C for one minute, annealing at 50°C for one minute, and primer elongation at 72°C for one minute. The cycling reaction was performed using a DNA Thermal Cycler (Perkin Elmer-Cetus, Norwalk, CT).

(d) Detection of the enriched single-stranded HCV sequence. The single-stranded enriched sequence was detected using a specific biotinylated probe and an anti-double stranded DNA antibody which is able to discriminate single-stranded from double-stranded DNA, *e.g.* target DNA hybridized or unhybridized with probe. The double-stranded DNA hybrids were visualized by generating a colorimetric signal.

One fourth of the enrichment reaction was denatured at 100°C for 5 min, then added to streptavidin-coated microtiter wells sensitized with 5 ng of biotinylated α-3CH probe (SEQ ID NO:7) and hybridized at 55°C for one hour. The immunochemical reaction was carried out as described in Example 1, above.

Eleven HCV-positive and four HCV-negative serum samples were analyzed by both LEDIA and PCR. The PCR reaction for detecting HCV was carried out as

follows. A reaction mixture was prepared in 100 μl containing dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 2 mM $MgCl_2$, 16 mM $NH_4(SO_4)_2$, 67 mM Tris Cl , pH 8.8, 50 pmoles of each primer 1CH (SEQ ID NO:14) and 2CH (SEQ ID NO:4), 50 units/ml of AmpliTaq (Perkin Elmer-Cetus, Norwalk, CT). To this mixture was added 1 μl of cDNA derived either from HCV positive patients or healthy blood donors, negative for any HCV marker. The PCR reaction was carried out for 45 cycles, each composed of three steps: a denaturation step at 94°C for 1 minute, an annealing step at 50°C for 1 minute, and an elongation step at 72°C for 1 minute. At the end of the reaction, an aliquot of 25 μl was analyzed using agarose gel electrophoresis. A band of amplified DNA measuring 299 base pairs was detected.

Table 7 compares the results of the LEDIA method with the PCR assay.

Table 7 - Comparison of LEDIA and PCR for HCV detection in serum samples

Serum Sample Code	LEDIA (OD 450 nm)*	PCR
33	> 3	+
34	0.107	-
35	> 3	+
36	> 3	+
37	> 3	+
38	0.983	+
39	0.069	-
BS	> 3	+
BC	1.195	+
BR	1.173	+
CS	0.104	-
CC	> 3	+
CR	2.441	+
BL	0.051	-
BS+	> 3	+

* cut off: 0.2 OD

This example demonstrates that the two methods, LEDIA and PCR, give equivalent results. Thus, linear enrichment of DNA and detection of single-stranded DNA using LEDIA yields the same performance as an exquisitely sensitive method such as PCR, using a single primer and a lower cycle number.

EXAMPLE 10

This example demonstrates the detection of hepatitis G viral sequences using the LEDIA method.

For PCR analysis, a 100 μl reaction mixture was prepared containing dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, $MgCl_2$ 2 mM, 16 mM $NH_4(SO_4)_2$, 67 mM Tris HCl, pH 8.8, 50 pmoles of each primer AC1-S (SEQ ID NO:12) and AC3-AS (SEQ ID NO:16), 50 units/ml of AmpliTaq (Perkin Elmer-Cetus, Norwalk, CT). To this mixture, 1 μl of cDNA derived either from HGV-2 positive patients or HGV-2 negative healthy blood donors was added, and the PCR reaction was carried

out for 45 cycles each composed of a denaturation step (94°C for 1 minute), an annealing step (52°C for 1 minute), and an elongation step (72°C for 1 minute). At the end of the reaction, an aliquot of 25 μl was subjected to agarose-gel electrophoresis. The PCR product was detected as an amplified DNA band of 422 base pairs.

One serum sample which was positive for hepatitis G sequences using PCR and one serum sample which was negative for hepatitis G sequences using PCR were analyzed using the LEDIA method, as described in Example 9, above, using 40 cycles of enrichment. The positive sample had an OD reading of >3, and the negative sample had an OD reading of 0.065.

Thus, LEDIA is capable of distinguishing hepatitis G-positive and hepatitis G-negative serum samples.

EXAMPLE 11

This example demonstrates detection of hepatitis B sequences in serum samples using LEDIA.

Hepatitis B DNA was extracted from 1 ml serum samples as follows. One milliliter of serum was digested for one hour at 56°C with 1 mg/ml proteinase K , in a total volume of 2 ml containing 0.01 M Tris Cl, pH 7.5, 0.01 M EDTA, 150 mM NaCl and 0.5 % sodium-dodecyl-sulfate (SDS). The sample was then extracted using one volume of a 1:1 solution of phenol-chloroform (BDH Laboratory Supplies, Poole, Dorset, UK), and the supernatant was precipitated by adding two volumes of cold ethanol and one-tenth volume of 3 M sodium acetate, pH 5.2. The pellet was washed twice with 75% cold ethanol, air-dried, and resuspended in 100 μl of H_2O.

The linear enrichment reaction was carried out as described in Example 4, above, using 40 cycles of enrichment. A serum sample containing a known amount of viral genomes (10^7 genomes/ml) was used as a positive control.

The single-stranded enriched hepatitis B sequence was detected using an antibody specific for double-stranded DNA, as described above.

EXAMPLE 12

This example demonstrates optimization of the linear enrichment reaction.

It is known that by extending the number of cycles, an exponential amplification of a target DNA may occur even if a single primer sequence is used. The degree and the cycle number at which this phenomenon starts can vary with different primers. It is believed that this behavior depends on enrichment of single-stranded DNA up to an amount such that the primer can weakly cross-react with enriched DNA in an a specific way and trigger the synthesis of a two-stranded template bearing the same primer site at both ends. This template causes a single-primer exponential amplification to occur.

Optimization of cycle number. The course of single-stranded enrichment vs double-stranded DNA exponential amplification was studied at different numbers of cycles. In order to determine the starting point of double-stranded DNA formation, 60 reaction cycles were carried out, and the DNA product was analyzed after every two cycles from cycle 30 to cycle 60. Double-stranded DNA formation was detected by hybridization of the reaction product with a biotinylated probe (3CH for hepatitis C, SEQ ID NO:6, and α-CD3 for hepatitis B, SEQ ID NO:11) complementary to the antisense or negative sequence in the case of HCV. The polynucleotide sequence shown in SEQ ID NO:15 was used as a positive control for α-3CH hybridization. The procedure employed for this purpose was the same as that described for single-strand enrichment detection, above.

In several replicate experiments, hepatitis C sequences were detected as single-stranded DNA starting from cycle 30, employing 1μl of a cDNA sample; from cycle 40 thereafter, exponential amplification occurred (Table 8).

Table 8 - LEDIA for HCV*. Course of linear enrichment vs exponential amplification (HCVB primer).

Cycle number primer HCVB (SEQ ID NO:2)	single strand signal° probe 3CH (SEQ ID NO:6)	second strand signal° probe α-3CH (SEQ ID NO:7)
30	0.043	0.023
32	0.087	0.025
34	0.219	0.024
36	0.516	0.050
38	1.842	0.140
40	> 3	1.805
42	> 3	> 3
44	> 3	> 3
46	> 3	> 3
48	> 3	> 3
50	> 3	> 3

* 2000 HCV genomes/reaction

° OD 450 nm, average of two replicates

Similar results were obtained in the case of hepatitis B (Table 9).

Table 9 - LEDIA for HBV. Course of linear enrichment vs exponential amplification (C2 primer, SEQ ID NO:8) with serial dilutions of target DNA.

Cycles number	HBV copy number	single strand signal* probe CD3 (SEQ ID NO:10)	second strand signal* probe α-CD3 (SEQ ID NO:11)
40	$1.5x10^5$	2.065	0.092
	$1.5x10^4$	2.609	0.051
	$1.5x10^3$	0.592	0.087
	$1.5x10^2$	0.364	0.074
45	$1.5x10^5$	2.119	0.364
	$1.5x10^4$	> 3	0.298
	$1.5x10^3$	1.874	0.254
	$1.5x10^2$	0.351	0.190
50	$1.5x10^5$	> 3	0.771
	$1.5x10^4$	> 3	0.748
	$1.5x10^3$	> 3	0.715
	$1.5x10^2$	1.050	0.708
55	$1.5x10^5$	2.508	0.740
	$1.5x10^4$	1.706	1.118
	$1.5x10^3$	> 3	1.104
	$1.5x10^2$	0.058	1.149
60	$1.5x10^5$	2.304	1.460
	$1.5x10^4$	1.811	1.478
	$1.5x10^3$	1.763	1.484
	$1.5x10^2$	2.548	1.177

Thus we fixed at 38 cycles (HCV) and 40 cycles (HBV) the optimal cycle number to obtain single-stranded DNA enrichment sufficient for single-stranded DNA analyte detection, at the same time avoiding exponential amplification which would overwhelm the linear reaction.

As it is shown in Tables 8 and 9, from cycle 50 to cycle 60, the signal began to decrease in some experiments and at some dilutions.

Table 10 - LEDIA for HCV* at different cycles number (primer HCVB, SEQ ID NO:2).

No. cycles	OD 450 nm probe 3CH (SEQ ID NO:6)
35	0.057
50	> 3
55	1.842
60	0.199

* $2x10^3$ HCV genomes/reaction

It is reasonable to assume that, as the contribution of double-stranded DNA to the final product becomes higher, a significant portion of single-stranded DNA (synthesized up to cycles around 40) is converted into double-stranded DNA. Therefore, even if the total amount of target DNA synthesized *de novo* becomes *higher* after exponential amplification takes place, in comparison to the condition in which only single-stranded DNA is produced, *less* DNA is actually amenable to hybridization. This data confirms that, in order to gain a better analytical performance, the linear enrichment of a target molecules is more suitable than its exponential amplification.

Primer sequence and concentration. Five different HCV primers (SEQ ID NOS:1-5) and one HBV primer (SEQ ID NO:8) were employed, each at different final concentration and at different temperatures. The relative contribution of each primer and of each primer concentration to the enrichment of single-stranded DNA analyte was studied.

Examples of the results observed with different primers, different primer concentrations and different temperatures are reported in Tables 11 and 12.

Table 11 - LEDIA for HCV*. Performances of different HCV primers at different concentrations

	Primer			
Primer pmoles/reaction	2CH SEQ ID NO:5	HCVA SEQ ID NO:1	HCVB SEQ ID NO: 2	HCVC SEQ ID NO:3
10	pos 0.036	0.042	> 3	0.045
	neg 0.035	0.032	0.113	0.043
25	pos 0.044	1.370	2.868	0.047
	neg 0.033	0.038	0.080	0.044
50	pos 0.035	2.477	> 3	0.641
	neg 0.036	0.035	0.097	0.103
100	pos 0.305	1.637	> 3	0.476
	neg 0.030	0.031	0.081	0.039

* Experimental conditions:
40 LEDIA cycles,
annealing temp. 50°C
$2x10^3$ HCV genomes/reaction

Table 12 - LEDIA for HCV detection in serum
Effect of temperature of annealing

	No. of cycles			
Temperature of annealing		45°C	50°C	55°C
Positive sample (OD)	34	0.409	0.538	0.174
	36	2.570	> 3	1.833
Negative sample (OD)	34	0.470	0.076	0.147
	36	0.284	0.036	0.366

From these data one can see that temperature of annealing, as expected, is an important parameter. However, parameters other than affinity for the target sequence, which drive the efficiency and specificity of polymerization, can be important in LEDIA. Generally, different primers perform in a dramatically different manner, and the optimal concentration may be very different among primers. Moreover, primers which perform well in PCR, such as those shown in SEQ ID NOS:14 and 5) can behave poorly in LEDIA, even in different combinations. Similarly, primers well-suited for LEDIA can perform poorly or not at all in PCR, such as the primers HCVA (SEQ ID NO:1) and HCVB (SEQ ID NO:2).

Primer selection and titration thus are critical steps for obtaining optimal performance using LEDIA. Also, the efficiency of LEDIA seems to be inversely proportional to the tendency to exponential amplification, of which primers are a critical component. As a whole, these data strongly suggest that parameters and thermodynamic constraints underlying the reaction are unique to LEDIA and are important features of the invention.

Sensitivity of the assay. Titration curves were performed on cDNA synthesized from serial two-fold dilutions of an HCV serum sample containing a known amount of HCV genome (500,000 genomes/ml) (one μl of cDNA corresponds to 1/20 of cDNA synthesized from 1/3 of 100 μl of serum). One microliter of cDNA derived from each dilution was processed by LEDIA. The last detectable dilution (10 target HCV sequences) gave an OD reading of 0.5, compared to a background signal of less than 0.1 OD.

TABLE 13 - Sensitivity of HCV LEDIA (HCVB primer, SEQ ID NO:2)*

HCV genome copy number	OD 450 nm
2000	> 3
1000	> 3
500	> 3
250	> 3
125	> 3
60	> 3
30	> 3
15	2.295
10	0.549
5	0.091
0	0.057

* 38 cycles, annealing 50°C

Specificity of the assay. To ascertain the specificity of LEDIA, several samples containing heterologous DNA, other than HCV-negative serum samples, were employed, including cDNA from HCV negative subjects infected with HGV, HBV or HIV; human genomic DNA, plasmids bearing human or HGV, HBV, HIV sequences. All these heterologous DNAs gave optical densities which were indistinguishable from background when processed by LEDIA. This data confirmed the specificity of the assay.

Use of different thermal cyclers. Different thermal cyclers may be based on different techniques for rapid temperature changes. We compared results obtained using a Perkin Elmer-Cetus DNA Thermal Cycler, in which rapid cooling is obtained by a refrigerator, with a RapidCycler from Idaho Technology, which is based on air convection to change temperatures, with excursion time shorter than Perkin Elmer Thermal Cycler. Both instruments gave closely similar results (see Table 10) and can be suitably used for LEDIA.

Use of different DNA polymerase enzymes. HCV cDNA was tested by LEDIA using three thermostable DNA polymerase preparations (AmpliTAq and GoldTaq Polymerase from Perkin Elmer-Cetus, and Pwo DNA polymerase from Boehringer Mannheim, Mannheim, Germany). The results are shown in Table 14 and demonstrate that different polymerases give similar performances using the LEDIA method.

Table 14 - LEDIA performed using different DNA polymerases and different thermal cyclers*

DNA polymerase (5 U/reaction)	OD 450 nm (HCV pos sample)		OD 450 nm (HCV neg sample)	
Thermal Cycler	PE	ID	PE	ID
AmpliTaq	> 3	> 3	0.068	0.057
GoldTaq	> 3	> 3	0.037	0.074
Pwo	> 3	> 3	0.025	0.029

Experimental conditions:
$1x10^3$ HCV genome copies
HCVB primer (SEQ ID NO:2), 100 pmoles/reaction
annealing 1 min 50 °C
38 reaction cycles

CLAIMS

1. A method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample, comprising the step of:

detecting a polynucleotide molecule on a solid support, wherein the polynucleotide molecule comprises (a) a single-stranded polynucleotide analyte and (b) one or more single-stranded polynucleotide probes hybridized to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded, wherein at least one of the single-stranded polynucleotide probes is bound to the solid support, wherein detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

2. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support prior to hyridization with the single-stranded polynucleotide analyte.

3. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support after hyridization with the single-stranded polynucleotide analyte.

4. The method of claim 1 wherein the single-stranded polynucleotide analyte is DNA.

5. The method of claim 1 wherein the one or more single-stranded polynucleotide probes are DNA probes.

6. The method of claim 5 wherein the DNA probe is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

7. The method of claim 5 wherein one or more of the DNA probes is 20-100 nucleotides in length.

8. The method of claim 5 wherein one or more of the DNA probes is less than 20 nucleotides in length.

9. The method of claim 5 wherein one or more of the DNA probes is less than 50 nucleotides in length.

10. The method of claim 5 wherein one or more of the DNA probes is less than 100 nucleotides in length.

11. The method of claim 4 wherein the first portion of the polynucleotide molecule is double-stranded DNA.

12. The method of claim 1 wherein the solid support is a particle.

13. The method of claim 5 wherein at least one single-stranded DNA probe which is not bound to the solid support comprises a segment which is double-stranded DNA.

14. The method of claim 4 wherein the biological sample is treated to form the single-stranded DNA analyte.

15. The method of claim 11 wherein the first portion of the polynucleotide molecule is detected using a reagent which specifically binds to double-stranded DNA.

16. The method of claim 15 wherein the reagent is a protein.

17. The method of claim 16 wherein the protein is a first antibody.

18. The method of claim 17 wherein the first antibody comprises a detectable label.

19. The method of claim 17 wherein specific binding of the first antibody to double-stranded DNA is detected by a second antibody which specifically binds to the first antibody.

20. The method of claim 17 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.3 pg/µl.

21. The method of claim 20 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 5 fg/µl.

22. The method of claim 21 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 1 fg/µl.

23. The method of claim 22 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.5 fg/µl.

24. The method of claim 23 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.1 fg/µl.

25. The method of claim 17 wherein the first antibody is a monoclonal antibody produced using spleen cells from an animal which is susceptible to autoimmune disease.

26. The method of claim 25 wherein the animal is an MRL/lpr mouse.

27. The method of claim 1 wherein the single-stranded polynucleotide probe comprises a first binding moiety and wherein the solid support comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other.

28. The method of claim 27 wherein the first or second binding moiety is biotin.

29. The method of claim 27 wherein the first or second binding moiety is avidin.

30. The method of claim 27 wherein the first or second binding moiety is streptavidin.

31. The method of claim 27 wherein the second binding moiety is an antibody.

32. The method of claim 1 wherein the single-stranded polynucleotide probe is covalently bound to the solid support.

33. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis C virus.

34. The method of claim 33 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

35. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis B virus.

36. The method of claim 35 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9.

37. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis G virus.

38. The method of claim 37 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:13.

39. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of an HIV virus.

40. The method of claim 39 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18.

41. The method of claim 1 wherein two or more distinct DNA probes are bound to the solid support.

42. The method of 42 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

43. The method of claim 42 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

44. The method of claim 1 wherein the polynucleotide molecule comprises a second portion which is single-stranded.

45. The method of claim 44 further comprising the step of:

synthesizing at least one polynucleotide strand which is complementary to all or a part of the second portion of the polynucleotide molecule to form additional double-stranded portions of the polynucleotide molecule.

46. The method of claim 45 wherein the step of synthesizing uses a non-thermostable polymerase.

47. The method of claim 46 wherein the non-thermostable polymerase is a T4 polymerase.

48. The method of claim 45 wherein the additional double-stranded portions of the polynucleotide molecule are double-stranded DNA.

49. The method of claim 48 wherein the additional double-stranded portions of the polynucleotide molecule are detected using an antibody which specifically binds to double-stranded DNA.

50. The method of claim 49 wherein the antibody comprises a detectable label.

51. The method of claim 4, further comprising the step of:

synthesizing additional copies of the single-stranded DNA analyte prior to hybridizing the single-stranded DNA analyte with the one or more single-stranded DNA probes.

52. The method of claim 51 wherein additional copies of the complement of the single-stranded DNA analyte are not synthesized.

53. The method of claim 51 wherein the step of synthesizing employs one single-stranded DNA primer.

54. The method of claim 51 wherein the step of synthesizing proceeds by linear kinetics.

55. The method of claim 51 wherein the single-stranded DNA primer is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 4, 5, 8, 10, and 12.

56. The method of claim 51 wherein the DNA probe is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

57. The method of claim 51 wherein the step of synthesizing uses a thermostable polymerase.

58. The method of claim 57 wherein the step of synthesizing uses fewer than 45 polymerization cycles.

59. The method of claim 58 wherein the step of synthesizing uses fewer than 30 polymerization cycles.

60. The method of claim 51 wherein two or more distinct single-stranded DNA probes are bound to the solid support.

61. The method of claim 60 wherein the step of synthesizing employs two or more distinct single-stranded DNA primers which are complementary to two or more distinct single-stranded DNA analytes.

62. The method of claim 51 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

63. The method of 62 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

64. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

at least one single-stranded DNA probe which comprises a first binding moiety;

a solid support which comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other; and

a monoclonal antibody which is capable of detecting double-stranded DNA.

65. The kit of claim 64 further comprising written instructions for a method comprising the step of:

detecting a DNA molecule on a solid support, wherein the DNA molecule comprises (a) a single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

66. The kit of claim 64 further comprising written instructions for a method comprising the steps of:

synthesizing additional copies of a single-stranded DNA analyte prior to hybridizing the single-stranded DNA analyte with one or more single-stranded DNA probes; and

detecting a DNA molecule on a solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

67. The kit of claim 64 wherein the solid support is a particle.

68. The kit of claim 64 which comprises two or more distinct DNA probes.

69. The kit of claim 66 further comprising a thermostable DNA polymerase.

70. The kit of claim 66 further comprising a single-stranded DNA primer for synthesizing additional copies of the single-stranded DNA analyte.

71. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

a solid support which comprises a single-stranded DNA probe; and

a monoclonal antibody which is capable of detecting double-stranded DNA.

72. The kit of claim 71 further comprising written instructions for a method comprising the step of:

detecting a DNA molecule on a solid support, wherein the DNA molecule comprises (a) a single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

73. The kit of claim 71 further comprising written instructions for a method comprising the steps of:

synthesizing additional copies of a single-stranded DNA analyte prior to hybridizing the single-stranded DNA analyte with one or more single-stranded DNA probes; and

detecting a DNA molecule on a solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

74. The kit of claim 71 wherein the solid support is a particle.

75. The kit of claim 71 wherein the solid support comprises two or more distinct DNA probes.

76. The kit of claim 73 further comprising a thermostable DNA polymerase.

77. The kit of claim 73 further comprising a single-stranded DNA primer for synthesizing additional copies of the single-stranded DNA analyte.

78. A single-stranded DNA primer which consists of a sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 5, 8, 10, and 12.

79. The method of claim 1 further comprising the step of:

quantitating the polynucleotide molecules on the solid support which comprise first portions which are double-stranded, wherein a quantity of the polynucleotide molecules is correlated with a quantity of the single-stranded polynucleotide analyte in the biological sample.

AMENDED CLAIMS

[received by the International Bureau on 17 May 1999 (17.05.99); original claims 1-79 replaced by new claims 1-119 (11 pages)]

1. A method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample, comprising the step of:

synthesizing additional copies of the single-stranded polynucleotide analyte, wherein additional copies of the complement of the single-stranded polynucleotide analyte are not synthesized; and

detecting a polynucleotide molecule on a solid support, wherein the polynucleotide molecule comprises (a) the single-stranded polynucleotide analyte and (b) one or more single-stranded polynucleotide probes hybridized to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded, wherein at least one of the single-stranded polynucleotide probes is bound to the solid support, wherein detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

2. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support prior to hybridization with the single-stranded polynucleotide analyte.

3. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support after hybridization with the single-stranded polynucleotide analyte.

4. The method of claim 1 wherein the single-stranded polynucleotide analyte is DNA.

5. The method of claim 1 wherein the one or more single-stranded polynucleotide probes are DNA probes.

6. The method of claim 5 wherein at least one of the one or more DNA [probe] probes is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

7. The method of claim 5 wherein one or more of the DNA probes is 20-100 nucleotides in length.

8. The method of claim 5 wherein one or more of the DNA probes is

less than 20 nucleotides in length.

9. The method of claim 5 wherein one or more of the DNA probes is less than 50 nucleotides in length.

10. The method of claim 5 wherein one or more of the DNA probes is less than 100 nucleotides in length.

11. The method of claim 5 wherein the first portion of the polynucleotide molecule is double-stranded DNA.

12. The method of claim 1 wherein the solid support is a particle.

13. The method of claim 5 wherein at least one single-stranded DNA probe which is not bound to the solid support comprises a segment which is double-stranded DNA.

14. The method of claim 4 wherein the biological sample is treated to form the single-stranded DNA analyte.

15. The method of claim 11 wherein the first portion of the polynucleotide molecule is detected using a reagent which specifically binds to double-stranded DNA.

16. The method of claim 15 wherein the reagent is a protein.

17. The method of claim 16 wherein the protein is a first antibody.

18. The method of claim 17 wherein the first antibody comprises a detectable label.

19. The method of claim 17 wherein specific binding of the first antibody to double-stranded DNA is detected by a second antibody which specifically binds to the first antibody.

20. The method of claim 17 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.3 pg/μl.

21. The method of claim 20 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 5 fg/μl.

22. The method of claim 21 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 1 fg/μl.

23. The method of claim 22 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.5 fg/μl.

24. The method of claim 23 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.1 fg/μl.

25. The method of claim 17 wherein the first antibody is a monoclonal antibody produced using spleen cells from an animal which is susceptible to autoimmune disease.

26. The method of claim 25 wherein the animal is an MRL/lpr mouse.

27. The method of claim 1 wherein the single-stranded polynucleotide probe comprises a first binding moiety and wherein the solid support comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other.

28. The method of claim 27 wherein the first or second binding moiety is biotin.

29. The method of claim 27 wherein the first or second binding moiety is avidin.

30. The method of claim 27 wherein the first or second binding moiety is streptavidin.

31. The method of claim 27 wherein the second binding moiety is an antibody.

32. The method of claim 1 wherein the single-stranded polynucleotide probe is covalently bound to the solid support.

33. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis C virus.

34. The method of claim 33 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

35. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis B virus.

36. The method of claim 35 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9.

37. The method of claim 1 wherein the single-stranded polynucleotide

probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis G virus.

38. The method of claim 37 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:13.

39. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of an HIV virus.

40. The method of claim 39 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18.

41. The method of claim 1 wherein two or more distinct DNA probes are bound to the solid support.

42. The method of 41 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

43. The method of claim 42 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

44. The method of claim 1 wherein the polynucleotide molecule comprises a second portion which is single-stranded.

45. The method of claim 44 further comprising the step of:

synthesizing at least one polynucleotide strand which is complementary to all or a part of the second portion of the polynucleotide molecule to form additional double-stranded portions of the polynucleotide molecule.

46. The method of claim 45 wherein the step of synthesizing uses a non-thermostable polymerase.

47. The method of claim 46 wherein the non-thermostable polymerase is a T4 polymerase.

48. The method of claim 45 wherein the additional double-stranded

portions of the polynucleotide molecule are double-stranded DNA.

49. The method of claim 48 wherein the additional double-stranded portions of the polynucleotide molecule are detected using an antibody which specifically binds to double-stranded DNA.

50. The method of claim 49 wherein the antibody comprises a detectable label.

51. The method of claim 1 wherein the step of synthesizing employs one single-stranded DNA primer.

52. The method of claim 1 wherein the step of synthesizing proceeds by linear kinetics.

53. The method of claim 1 wherein the single-stranded DNA primer is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 4, 5, 8, 10, and 12.

54. The method of claim 1 wherein the DNA probe is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

55. The method of claim 1 wherein the step of synthesizing uses a thermostable polymerase.

56. The method of claim 55 wherein the step of synthesizing uses fewer than 45 polymerization cycles.

57. The method of claim 56 wherein the step of synthesizing uses fewer than 30 polymerization cycles.

58. The method of claim 1 wherein two or more distinct single-stranded DNA probes are bound to the solid support.

59. The method of claim 58 wherein the step of synthesizing employs two or more distinct single-stranded DNA primers which are complementary to two or more distinct single-stranded DNA analytes.

60. The method of claim 1 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is

complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

61. The method of 60 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

62. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

at least one single-stranded DNA probe which comprises a first binding moiety;

a solid support which comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other; [and]

a monoclonal antibody which is capable of detecting double-stranded DNA; and

written instructions for a method comprising the steps of:

synthesizing additional copies of the single-stranded DNA analyte prior to hybridizing the single-stranded DNA analyte with the at least one single-stranded DNA probe, wherein additional copies of the complement of the single-stranded polynucleotide analyte are not synthesized; and

detecting a DNA molecule on the solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) at least one of the single-stranded DNA probes which is specifically hybridized to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein the at least one single-stranded DNA probe is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

63. The kit of claim 62 wherein the solid support is a particle.

64. The kit of claim 62 which comprises two or more distinct DNA probes.

65. The kit of claim 62 further comprising a thermostable DNA polymerase.

66. The kit of claim 62 further comprising a single-stranded DNA primer for synthesizing additional copies of the single-stranded DNA analyte.

67. A single-stranded DNA primer which consists of a sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 5, 8, 10, and 12.

68. The method of claim 1 further comprising the step of:

quantitating the polynucleotide molecules on the solid support which comprise first portions which are double-stranded, wherein a quantity of the polynucleotide molecules is correlated with a quantity of the single-stranded polynucleotide analyte in the biological sample.

69. A method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample, comprising the step of:

detecting a polynucleotide molecule on a solid support, wherein the polynucleotide molecule comprises (a) the single-stranded polynucleotide analyte and (b) one or more single-stranded polynucleotide probes hybridized to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded, wherein at least one of the single-stranded polynucleotide probes is bound to the solid support after hybridization with the single-stranded polynucleotide analyte, wherein detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

70. The method of claim 69 wherein the single-stranded polynucleotide analyte is DNA.

71. The method of claim 69 wherein the one or more single-stranded polynucleotide probes are DNA probes.

72. The method of claim 71 wherein at least one of the one or more DNA probes is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

73. The method of claim 71 wherein one or more of the DNA probes is 20-100 nucleotides in length.

74. The method of claim 71 wherein one or more of the DNA probes is

less than 20 nucleotides in length.

75. The method of claim 71 wherein one or more of the DNA probes is less than 50 nucleotides in length.

76. The method of claim 71 wherein one or more of the DNA probes is less than 100 nucleotides in length.

77. The method of claim 69 wherein the first portion of the polynucleotide molecule is double-stranded DNA.

78. The method of claim 69 wherein the solid support is a particle.

79. The method of claim 71 wherein at least one single-stranded DNA probe which is not bound to the solid support comprises a segment which is double-stranded DNA.

80. The method of claim 69 wherein the biological sample is treated to form the single-stranded DNA analyte.

81. The method of claim 69 wherein the first portion of the polynucleotide molecule is detected using a reagent which specifically binds to double-stranded DNA.

82. The method of claim 81 wherein the reagent is a protein.

83. The method of claim 82 wherein the protein is a first antibody.

84. The method of claim 83 wherein the first antibody comprises a detectable label.

85. The method of claim 83 wherein specific binding of the first antibody to double-stranded DNA is detected by a second antibody which specifically binds to the first antibody.

86. The method of claim 69 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.3 pg/μl.

87. The method of claim 86 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 5 fg/μl.

88. The method of claim 87 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 1 fg/μl.

89. The method of claim 88 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.5 fg/μl.

90. The method of claim 89 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.1 fg/μl.

91. The method of claim 83 wherein the first antibody is a monoclonal antibody produced using spleen cells from an animal which is susceptible to autoimmune disease.

92. The method of claim 91 wherein the animal is an MRL/1pr mouse.

93. The method of claim 69 wherein the single-stranded polynucleotide probe comprises a first binding moiety and wherein the solid support comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other.

94. The method of claim 93 wherein the first or second binding moiety is biotin.

95. The method of claim 93 wherein the first or second binding moiety is avidin.

96. The method of claim 93 wherein the first or second binding moiety is streptavidin.

97. The method of claim 93 wherein the second binding moiety is an antibody.

98. The method of claim 69 wherein the single-stranded polynucleotide probe is covalently bound to the solid support.

99. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis C virus.

100. The method of claim 99 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

101. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis B virus.

102. The method of claim 101 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9.

103. The method of claim 69 wherein the single-stranded polynucleotide

probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis G virus.

104. The method of claim 103 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:13.

105. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of an HIV virus.

106. The method of claim 105 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18.

107. The method of claim 69 wherein the polynucleotide molecule comprises a second portion which is single-stranded.

108. The method of claim 69 further comprising the step of:

synthesizing at least one polynucleotide strand which is complementary to all or a part of the second portion of the polynucleotide molecule to form additional double-stranded portions of the polynucleotide molecule.

109. The method of claim 69 further comprising the step of coupling additional double-stranded DNA to the polynucleotide molecule.

110. The method of 109 wherein a dendrimer is coupled to the polynucleotide molecule.

111. The method of claim 108 wherein the step of synthesizing uses a non-thermostable polymerase.

112. The method of claim 111 wherein the non-thermostable polymerase is a T4 polymerase.

113. The method of claim 108 wherein the additional double-stranded portions of the polynucleotide molecule are double-stranded DNA.

114. The method of claim 108 wherein the additional double-stranded portions of the polynucleotide molecule are detected using an antibody which specifically binds to double-stranded DNA.

115. The method of claim 114 wherein the antibody comprises a detectable label.

116. The method of claim 69 wherein two or more distinct single-stranded

DNA probes are used.

117. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

at least one single-stranded DNA probe which comprises a first binding moiety;

a solid support which comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other; [and]

a monoclonal antibody which is capable of detecting double-stranded DNA; and

written instructions for a method comprising the step of:

detecting a DNA molecule on the solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support after hybridization with the single-stranded polynucleotide analyte, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

118. The kit of claim 117 wherein the solid support is a particle.

119. The kit of claim 117 which comprises two or more distinct DNA probes.

FIG. 1
CMV PARTICLES

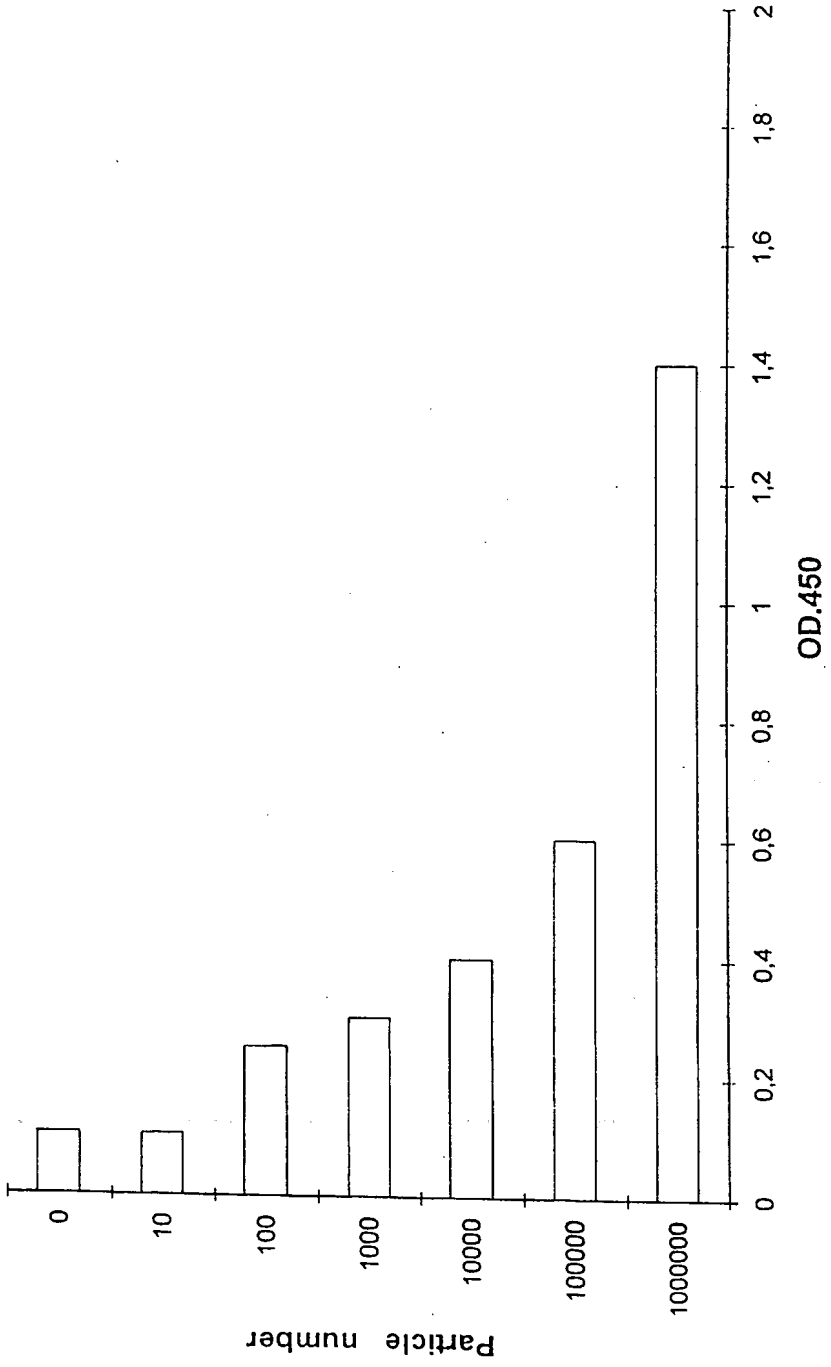


FIG. 2

COMPARISON OF AIDA WITH (■) AND WITHOUT (▲) T4 POLYMERASE

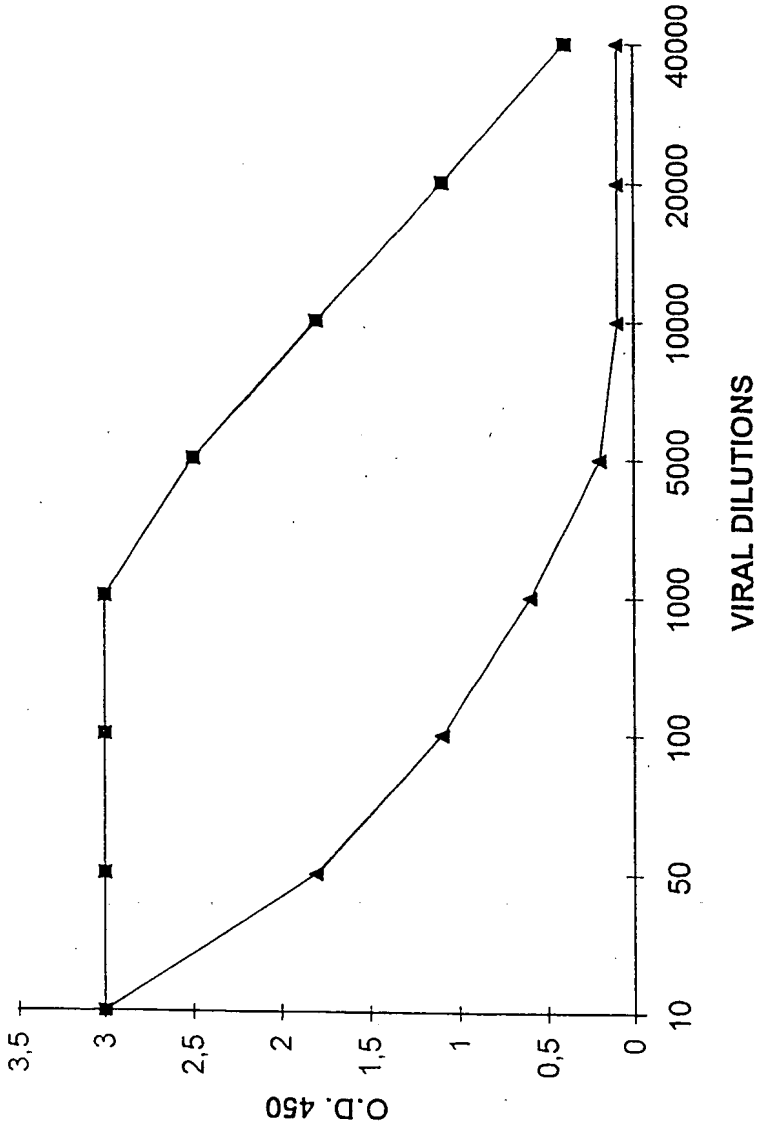
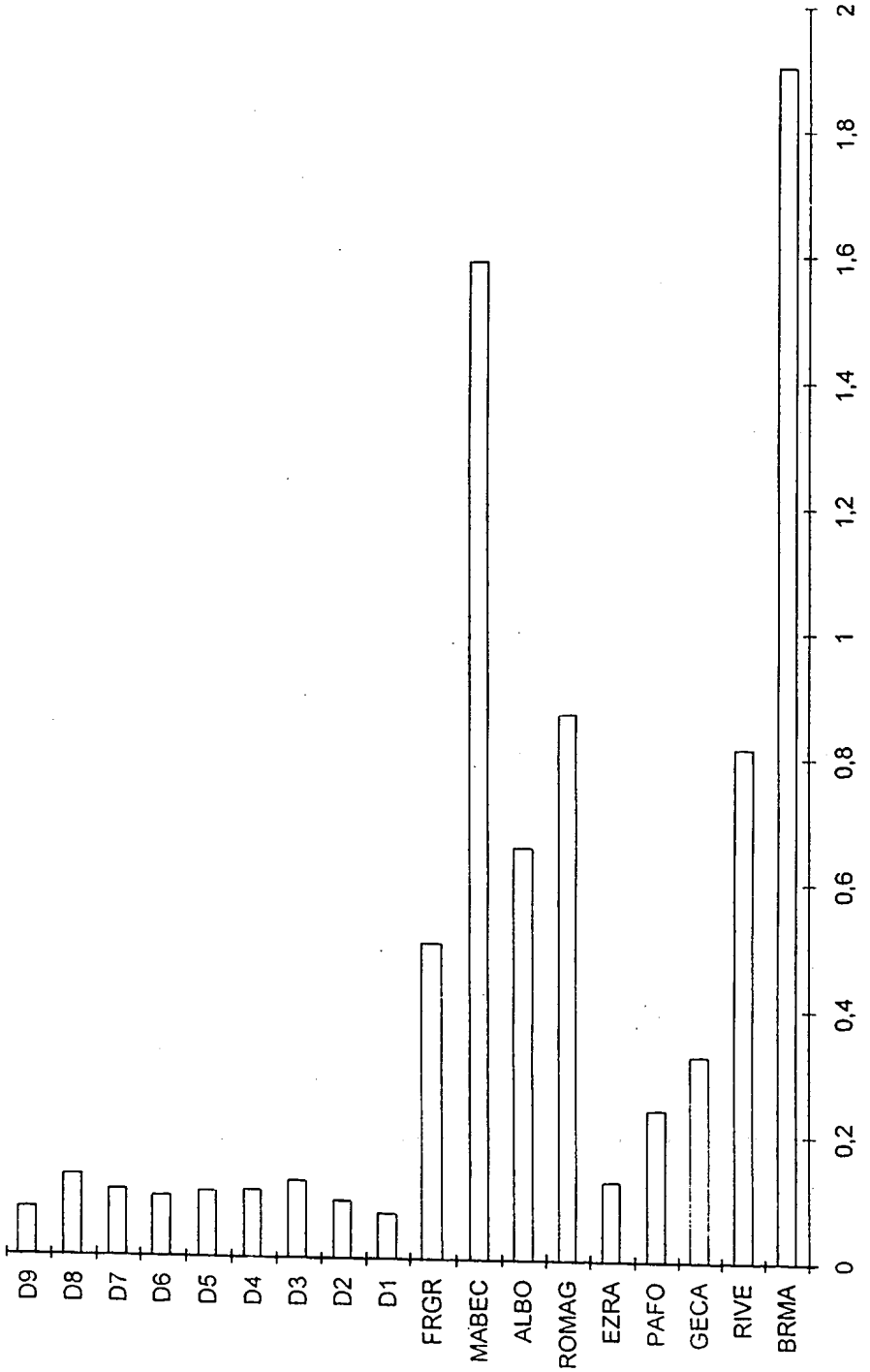


FIG. 3
AIDA-HIV



SEQUENCE LISTING

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<213> Hepatitis C virus

<400> 3

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tcctttct 68

<210> 7

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gctctccc 68

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<210> 21
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<213> Hepatitis C virus

<400> 21

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CLAIMS

1. A method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample, comprising the step of:

synthesizing additional copies of the single-stranded polynucleotide analyte, wherein additional copies of the complement of the single-stranded polynucleotide analyte are not synthesized; and

detecting a polynucleotide molecule on a solid support, wherein the polynucleotide molecule comprises (a) the single-stranded polynucleotide analyte and (b) one or more single-stranded polynucleotide probes hybridized to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded, wherein at least one of the single-stranded polynucleotide probes is bound to the solid support, wherein detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

2. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support prior to hybridization with the single-stranded polynucleotide analyte.

3. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support after hybridization with the single-stranded polynucleotide analyte.

4. The method of claim 1 wherein the single-stranded polynucleotide analyte is DNA.

5. The method of claim 1 wherein the one or more single-stranded polynucleotide probes are DNA probes.

6. The method of claim 5 wherein <u>at least one of</u> the <u>one or more</u> DNA [probe] <u>probes</u> is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

7. The method of claim 5 wherein one or more of the DNA probes is 20-100 nucleotides in length.

8. The method of claim 5 wherein one or more of the DNA probes is

less than 20 nucleotides in length.

9. The method of claim 5 wherein one or more of the DNA probes is less than 50 nucleotides in length.

10. The method of claim 5 wherein one or more of the DNA probes is less than 100 nucleotides in length.

11. The method of claim 5 wherein the first portion of the polynucleotide molecule is double-stranded DNA.

12. The method of claim 1 wherein the solid support is a particle.

13. The method of claim 5 wherein at least one single-stranded DNA probe which is not bound to the solid support comprises a segment which is double-stranded DNA.

14. The method of claim 4 wherein the biological sample is treated to form the single-stranded DNA analyte.

15. The method of claim 11 wherein the first portion of the polynucleotide molecule is detected using a reagent which specifically binds to double-stranded DNA.

16. The method of claim 15 wherein the reagent is a protein.

17. The method of claim 16 wherein the protein is a first antibody.

18. The method of claim 17 wherein the first antibody comprises a detectable label.

19. The method of claim 17 wherein specific binding of the first antibody to double-stranded DNA is detected by a second antibody which specifically binds to the first antibody.

20. The method of claim 17 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.3 pg/μl.

21. The method of claim 20 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 5 fg/μl.

22. The method of claim 21 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 1 fg/μl.

23. The method of claim 22 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.5 fg/μl.

24. The method of claim 23 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.1 fg/μl.

25. The method of claim 17 wherein the first antibody is a monoclonal antibody produced using spleen cells from an animal which is susceptible to autoimmune disease.

26. The method of claim 25 wherein the animal is an MRL/lpr mouse.

27. The method of claim 1 wherein the single-stranded polynucleotide probe comprises a first binding moiety and wherein the solid support comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other.

28. The method of claim 27 wherein the first or second binding moiety is biotin.

29. The method of claim 27 wherein the first or second binding moiety is avidin.

30. The method of claim 27 wherein the first or second binding moiety is streptavidin.

31. The method of claim 27 wherein the second binding moiety is an antibody.

32. The method of claim 1 wherein the single-stranded polynucleotide probe is covalently bound to the solid support.

33. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis C virus.

34. The method of claim 33 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

35. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis B virus.

36. The method of claim 35 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9.

37. The method of claim 1 wherein the single-stranded polynucleotide

probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis G virus.

38. The method of claim 37 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:13.

39. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of an HIV virus.

40. The method of claim 39 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18.

41. The method of claim 1 wherein two or more distinct DNA probes are bound to the solid support.

42. The method of 41 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

43. The method of claim 42 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

44. The method of claim 1 wherein the polynucleotide molecule comprises a second portion which is single-stranded.

45. The method of claim 44 further comprising the step of:

synthesizing at least one polynucleotide strand which is complementary to all or a part of the second portion of the polynucleotide molecule to form additional double-stranded portions of the polynucleotide molecule.

46. The method of claim 45 wherein the step of synthesizing uses a non-thermostable polymerase.

47. The method of claim 46 wherein the non-thermostable polymerase is a T4 polymerase.

48. The method of claim 45 wherein the additional double-stranded

portions of the polynucleotide molecule are double-stranded DNA.

49. The method of claim 48 wherein the additional double-stranded portions of the polynucleotide molecule are detected using an antibody which specifically binds to double-stranded DNA.

50. The method of claim 49 wherein the antibody comprises a detectable label.

51. The method of claim 1 wherein the step of synthesizing employs one single-stranded DNA primer.

52. The method of claim 1 wherein the step of synthesizing proceeds by linear kinetics.

53. The method of claim 1 wherein the single-stranded DNA primer is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 4, 5, 8, 10, and 12.

54. The method of claim 1 wherein the DNA probe is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

55. The method of claim 1 wherein the step of synthesizing uses a thermostable polymerase.

56. The method of claim 55 wherein the step of synthesizing uses fewer than 45 polymerization cycles.

57. The method of claim 56 wherein the step of synthesizing uses fewer than 30 polymerization cycles.

58. The method of claim 1 wherein two or more distinct single-stranded DNA probes are bound to the solid support.

59. The method of claim 58 wherein the step of synthesizing employs two or more distinct single-stranded DNA primers which are complementary to two or more distinct single-stranded DNA analytes.

60. The method of claim 1 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is

complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

61. The method of 60 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

62. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

at least one single-stranded DNA probe which comprises a first binding moiety;

a solid support which comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other; [and]

a monoclonal antibody which is capable of detecting double-stranded DNA; and

written instructions for a method comprising the steps of:

synthesizing additional copies of the single-stranded DNA analyte prior to hybridizing the single-stranded DNA analyte with the at least one single-stranded DNA probe, wherein additional copies of the complement of the single-stranded polynucleotide analyte are not synthesized; and

detecting a DNA molecule on the solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) at least one of the single-stranded DNA probes which is specifically hybridized to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein the at least one single-stranded DNA probe is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

63. The kit of claim 62 wherein the solid support is a particle.

64. The kit of claim 62 which comprises two or more distinct DNA probes.

65. The kit of claim 62 further comprising a thermostable DNA polymerase.

66. The kit of claim 62 further comprising a single-stranded DNA primer for synthesizing additional copies of the single-stranded DNA analyte.

67. A single-stranded DNA primer which consists of a sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 5, 8, 10, and 12.

68. The method of claim 1 further comprising the step of:

quantitating the polynucleotide molecules on the solid support which comprise first portions which are double-stranded, wherein a quantity of the polynucleotide molecules is correlated with a quantity of the single-stranded polynucleotide analyte in the biological sample.

69. A method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample, comprising the step of:

detecting a polynucleotide molecule on a solid support, wherein the polynucleotide molecule comprises (a) the single-stranded polynucleotide analyte and (b) one or more single-stranded polynucleotide probes hybridized to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded, wherein at least one of the single-stranded polynucleotide probes is bound to the solid support after hybridization with the single-stranded polynucleotide analyte, wherein detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

70. The method of claim 69 wherein the single-stranded polynucleotide analyte is DNA.

71. The method of claim 69 wherein the one or more single-stranded polynucleotide probes are DNA probes.

72. The method of claim 71 wherein at least one of the one or more DNA probes is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

73. The method of claim 71 wherein one or more of the DNA probes is 20-100 nucleotides in length.

74. The method of claim 71 wherein one or more of the DNA probes is

less than 20 nucleotides in length.

75. The method of claim 71 wherein one or more of the DNA probes is less than 50 nucleotides in length.

76. The method of claim 71 wherein one or more of the DNA probes is less than 100 nucleotides in length.

77. The method of claim 69 wherein the first portion of the polynucleotide molecule is double-stranded DNA.

78. The method of claim 69 wherein the solid support is a particle.

79. The method of claim 71 wherein at least one single-stranded DNA probe which is not bound to the solid support comprises a segment which is double-stranded DNA.

80. The method of claim 69 wherein the biological sample is treated to form the single-stranded DNA analyte.

81. The method of claim 69 wherein the first portion of the polynucleotide molecule is detected using a reagent which specifically binds to double-stranded DNA.

82. The method of claim 81 wherein the reagent is a protein.

83. The method of claim 82 wherein the protein is a first antibody.

84. The method of claim 83 wherein the first antibody comprises a detectable label.

85. The method of claim 83 wherein specific binding of the first antibody to double-stranded DNA is detected by a second antibody which specifically binds to the first antibody.

86. The method of claim 69 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.3 pg/μl.

87. The method of claim 86 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 5 fg/μl.

88. The method of claim 87 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 1 fg/μl.

89. The method of claim 88 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.5 fg/μl.

90. The method of claim 89 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.1 fg/μl.

91. The method of claim 83 wherein the first antibody is a monoclonal antibody produced using spleen cells from an animal which is susceptible to autoimmune disease.

92. The method of claim 91 wherein the animal is an MRL/lpr mouse.

93. The method of claim 69 wherein the single-stranded polynucleotide probe comprises a first binding moiety and wherein the solid support comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other.

94. The method of claim 93 wherein the first or second binding moiety is biotin.

95. The method of claim 93 wherein the first or second binding moiety is avidin.

96. The method of claim 93 wherein the first or second binding moiety is streptavidin.

97. The method of claim 93 wherein the second binding moiety is an antibody.

98. The method of claim 69 wherein the single-stranded polynucleotide probe is covalently bound to the solid support.

99. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis C virus.

100. The method of claim 99 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

101. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis B virus.

102. The method of claim 101 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9.

103. The method of claim 69 wherein the single-stranded polynucleotide

probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis G virus.

104. The method of claim 103 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:13.

105. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of an HIV virus.

106. The method of claim 105 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18.

107. The method of claim 69 wherein the polynucleotide molecule comprises a second portion which is single-stranded.

108. The method of claim 69 further comprising the step of:

synthesizing at least one polynucleotide strand which is complementary to all or a part of the second portion of the polynucleotide molecule to form additional double-stranded portions of the polynucleotide molecule.

109. The method of claim 69 further comprising the step of coupling additional double-stranded DNA to the polynucleotide molecule.

110. The method of 109 wherein a dendrimer is coupled to the polynucleotide molecule.

111. The method of claim 108 wherein the step of synthesizing uses a non-thermostable polymerase.

112. The method of claim 111 wherein the non-thermostable polymerase is a T4 polymerase.

113. The method of claim 108 wherein the additional double-stranded portions of the polynucleotide molecule are double-stranded DNA.

114. The method of claim 108 wherein the additional double-stranded portions of the polynucleotide molecule are detected using an antibody which specifically binds to double-stranded DNA.

115. The method of claim 114 wherein the antibody comprises a detectable label.

116. The method of claim 69 wherein two or more distinct single-stranded

DNA probes are used.

117. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

at least one single-stranded DNA probe which comprises a first binding moiety;

a solid support which comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other; [and]

a monoclonal antibody which is capable of detecting double-stranded DNA; and

written instructions for a method comprising the step of:

detecting a DNA molecule on the solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support after hybridization with the single-stranded polynucleotide analyte, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

118. The kit of claim 117 wherein the solid support is a particle.

119. The kit of claim 117 which comprises two or more distinct DNA probes.

AMENDED CLAIMS

[received by the International Bureau on 17 May 1999 (17.05.99); original claims 1-79 replaced by new claims 1-119 (11 pages)]

1. A method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample, comprising the step of:

synthesizing additional copies of the single-stranded polynucleotide analyte, wherein additional copies of the complement of the single-stranded polynucleotide analyte are not synthesized; and

detecting a polynucleotide molecule on a solid support, wherein the polynucleotide molecule comprises (a) the single-stranded polynucleotide analyte and (b) one or more single-stranded polynucleotide probes hybridized to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded, wherein at least one of the single-stranded polynucleotide probes is bound to the solid support, wherein detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

2. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support prior to hybridization with the single-stranded polynucleotide analyte.

3. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support after hybridization with the single-stranded polynucleotide analyte.

4. The method of claim 1 wherein the single-stranded polynucleotide analyte is DNA.

5. The method of claim 1 wherein the one or more single-stranded polynucleotide probes are DNA probes.

6. The method of claim 5 wherein <u>at least one of</u> the <u>one or more</u> DNA [probe] <u>probes</u> is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

7. The method of claim 5 wherein one or more of the DNA probes is 20-100 nucleotides in length.

8. The method of claim 5 wherein one or more of the DNA probes is

less than 20 nucleotides in length.

9. The method of claim 5 wherein one or more of the DNA probes is less than 50 nucleotides in length.

10. The method of claim 5 wherein one or more of the DNA probes is less than 100 nucleotides in length.

11. The method of claim 5 wherein the first portion of the polynucleotide molecule is double-stranded DNA.

12. The method of claim 1 wherein the solid support is a particle.

13. The method of claim 5 wherein at least one single-stranded DNA probe which is not bound to the solid support comprises a segment which is double-stranded DNA.

14. The method of claim 4 wherein the biological sample is treated to form the single-stranded DNA analyte.

15. The method of claim 11 wherein the first portion of the polynucleotide molecule is detected using a reagent which specifically binds to double-stranded DNA.

16. The method of claim 15 wherein the reagent is a protein.

17. The method of claim 16 wherein the protein is a first antibody.

18. The method of claim 17 wherein the first antibody comprises a detectable label.

19. The method of claim 17 wherein specific binding of the first antibody to double-stranded DNA is detected by a second antibody which specifically binds to the first antibody.

20. The method of claim 17 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.3 pg/μl.

21. The method of claim 20 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 5 fg/μl.

22. The method of claim 21 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 1 fg/μl.

23. The method of claim 22 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.5 fg/μl.

24. The method of claim 23 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.1 fg/μl.

25. The method of claim 17 wherein the first antibody is a monoclonal antibody produced using spleen cells from an animal which is susceptible to autoimmune disease.

26. The method of claim 25 wherein the animal is an MRL/lpr mouse.

27. The method of claim 1 wherein the single-stranded polynucleotide probe comprises a first binding moiety and wherein the solid support comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other.

28. The method of claim 27 wherein the first or second binding moiety is biotin.

29. The method of claim 27 wherein the first or second binding moiety is avidin.

30. The method of claim 27 wherein the first or second binding moiety is streptavidin.

31. The method of claim 27 wherein the second binding moiety is an antibody.

32. The method of claim 1 wherein the single-stranded polynucleotide probe is covalently bound to the solid support.

33. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis C virus.

34. The method of claim 33 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

35. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis B virus.

36. The method of claim 35 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9.

37. The method of claim 1 wherein the single-stranded polynucleotide

probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis G virus.

38. The method of claim 37 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:13.

39. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of an HIV virus.

40. The method of claim 39 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18.

41. The method of claim 1 wherein two or more distinct DNA probes are bound to the solid support.

42. The method of 41 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

43. The method of claim 42 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

44. The method of claim 1 wherein the polynucleotide molecule comprises a second portion which is single-stranded.

45. The method of claim 44 further comprising the step of:

synthesizing at least one polynucleotide strand which is complementary to all or a part of the second portion of the polynucleotide molecule to form additional double-stranded portions of the polynucleotide molecule.

46. The method of claim 45 wherein the step of synthesizing uses a non-thermostable polymerase.

47. The method of claim 46 wherein the non-thermostable polymerase is a T4 polymerase.

48. The method of claim 45 wherein the additional double-stranded

portions of the polynucleotide molecule are double-stranded DNA.

49. The method of claim 48 wherein the additional double-stranded portions of the polynucleotide molecule are detected using an antibody which specifically binds to double-stranded DNA.

50. The method of claim 49 wherein the antibody comprises a detectable label.

51. The method of claim 1 wherein the step of synthesizing employs one single-stranded DNA primer.

52. The method of claim 1 wherein the step of synthesizing proceeds by linear kinetics.

53. The method of claim 1 wherein the single-stranded DNA primer is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 4, 5, 8, 10, and 12.

54. The method of claim 1 wherein the DNA probe is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

55. The method of claim 1 wherein the step of synthesizing uses a thermostable polymerase.

56. The method of claim 55 wherein the step of synthesizing uses fewer than 45 polymerization cycles.

57. The method of claim 56 wherein the step of synthesizing uses fewer than 30 polymerization cycles.

58. The method of claim 1 wherein two or more distinct single-stranded DNA probes are bound to the solid support.

59. The method of claim 58 wherein the step of synthesizing employs two or more distinct single-stranded DNA primers which are complementary to two or more distinct single-stranded DNA analytes.

60. The method of claim 1 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is

complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

61. The method of 60 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

62. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

at least one single-stranded DNA probe which comprises a first binding moiety;

a solid support which comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other; [and]

a monoclonal antibody which is capable of detecting double-stranded DNA; and

written instructions for a method comprising the steps of:

synthesizing additional copies of the single-stranded DNA analyte prior to hybridizing the single-stranded DNA analyte with the at least one single-stranded DNA probe, wherein additional copies of the complement of the single-stranded polynucleotide analyte are not synthesized; and

detecting a DNA molecule on the solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) at least one of the single-stranded DNA probes which is specifically hybridized to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein the at least one single-stranded DNA probe is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

63. The kit of claim 62 wherein the solid support is a particle.

64. The kit of claim 62 which comprises two or more distinct DNA probes.

65. The kit of claim 62 further comprising a thermostable DNA polymerase.

66. The kit of claim 62 further comprising a single-stranded DNA primer for synthesizing additional copies of the single-stranded DNA analyte.

67. A single-stranded DNA primer which consists of a sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 5, 8, 10, and 12.

68. The method of claim 1 further comprising the step of:

quantitating the polynucleotide molecules on the solid support which comprise first portions which are double-stranded, wherein a quantity of the polynucleotide molecules is correlated with a quantity of the single-stranded polynucleotide analyte in the biological sample.

69. A method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample, comprising the step of:

detecting a polynucleotide molecule on a solid support, wherein the polynucleotide molecule comprises (a) the single-stranded polynucleotide analyte and (b) one or more single-stranded polynucleotide probes hybridized to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded, wherein at least one of the single-stranded polynucleotide probes is bound to the solid support after hybridization with the single-stranded polynucleotide analyte, wherein detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

70. The method of claim 69 wherein the single-stranded polynucleotide analyte is DNA.

71. The method of claim 69 wherein the one or more single-stranded polynucleotide probes are DNA probes.

72. The method of claim 71 wherein at least one of the one or more DNA probes is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

73. The method of claim 71 wherein one or more of the DNA probes is 20-100 nucleotides in length.

74. The method of claim 71 wherein one or more of the DNA probes is

less than 20 nucleotides in length.

75. The method of claim 71 wherein one or more of the DNA probes is less than 50 nucleotides in length.

76. The method of claim 71 wherein one or more of the DNA probes is less than 100 nucleotides in length.

77. The method of claim 69 wherein the first portion of the polynucleotide molecule is double-stranded DNA.

78. The method of claim 69 wherein the solid support is a particle.

79. The method of claim 71 wherein at least one single-stranded DNA probe which is not bound to the solid support comprises a segment which is double-stranded DNA.

80. The method of claim 69 wherein the biological sample is treated to form the single-stranded DNA analyte.

81. The method of claim 69 wherein the first portion of the polynucleotide molecule is detected using a reagent which specifically binds to double-stranded DNA.

82. The method of claim 81 wherein the reagent is a protein.

83. The method of claim 82 wherein the protein is a first antibody.

84. The method of claim 83 wherein the first antibody comprises a detectable label.

85. The method of claim 83 wherein specific binding of the first antibody to double-stranded DNA is detected by a second antibody which specifically binds to the first antibody.

86. The method of claim 69 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.3 pg/μl.

87. The method of claim 86 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 5 fg/μl.

88. The method of claim 87 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 1 fg/μl.

89. The method of claim 88 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.5 fg/μl.

90. The method of claim 89 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.1 fg/μl.

91. The method of claim 83 wherein the first antibody is a monoclonal antibody produced using spleen cells from an animal which is susceptible to autoimmune disease.

92. The method of claim 91 wherein the animal is an MRL/lpr mouse.

93. The method of claim 69 wherein the single-stranded polynucleotide probe comprises a first binding moiety and wherein the solid support comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other.

94. The method of claim 93 wherein the first or second binding moiety is biotin.

95. The method of claim 93 wherein the first or second binding moiety is avidin.

96. The method of claim 93 wherein the first or second binding moiety is streptavidin.

97. The method of claim 93 wherein the second binding moiety is an antibody.

98. The method of claim 69 wherein the single-stranded polynucleotide probe is covalently bound to the solid support.

99. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis C virus.

100. The method of claim 99 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

101. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis B virus.

102. The method of claim 101 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9.

103. The method of claim 69 wherein the single-stranded polynucleotide

probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis G virus.

104. The method of claim 103 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:13.

105. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of an HIV virus.

106. The method of claim 105 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18.

107. The method of claim 69 wherein the polynucleotide molecule comprises a second portion which is single-stranded.

108. The method of claim 69 further comprising the step of:

synthesizing at least one polynucleotide strand which is complementary to all or a part of the second portion of the polynucleotide molecule to form additional double-stranded portions of the polynucleotide molecule.

109. The method of claim 69 further comprising the step of coupling additional double-stranded DNA to the polynucleotide molecule.

110. The method of 109 wherein a dendrimer is coupled to the polynucleotide molecule.

111. The method of claim 108 wherein the step of synthesizing uses a non-thermostable polymerase.

112. The method of claim 111 wherein the non-thermostable polymerase is a T4 polymerase.

113. The method of claim 108 wherein the additional double-stranded portions of the polynucleotide molecule are double-stranded DNA.

114. The method of claim 108 wherein the additional double-stranded portions of the polynucleotide molecule are detected using an antibody which specifically binds to double-stranded DNA.

115. The method of claim 114 wherein the antibody comprises a detectable label.

116. The method of claim 69 wherein two or more distinct single-stranded

DNA probes are used.

117. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

at least one single-stranded DNA probe which comprises a first binding moiety;

a solid support which comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other; [and]

a monoclonal antibody which is capable of detecting double-stranded DNA; and

written instructions for a method comprising the step of:

detecting a DNA molecule on the solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support after hybridization with the single-stranded polynucleotide analyte, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

118. The kit of claim 117 wherein the solid support is a particle.

119. The kit of claim 117 which comprises two or more distinct DNA probes.

CLAIMS

1. A method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample, comprising the step of:

detecting a polynucleotide molecule on a solid support, wherein the polynucleotide molecule comprises (a) a single-stranded polynucleotide analyte and (b) one or more single-stranded polynucleotide probes hybridized to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded, wherein at least one of the single-stranded polynucleotide probes is bound to the solid support, wherein detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

2. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support prior to hyridization with the single-stranded polynucleotide analyte.

3. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support after hyridization with the single-stranded polynucleotide analyte.

4. The method of claim 1 wherein the single-stranded polynucleotide analyte is DNA.

5. The method of claim 1 wherein the one or more single-stranded polynucleotide probes are DNA probes.

6. The method of claim 5 wherein the DNA probe is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

7. The method of claim 5 wherein one or more of the DNA probes is 20-100 nucleotides in length.

8. The method of claim 5 wherein one or more of the DNA probes is less than 20 nucleotides in length.

9. The method of claim 5 wherein one or more of the DNA probes is less than 50 nucleotides in length.

10. The method of claim 5 wherein one or more of the DNA probes is less than 100 nucleotides in length.

11. The method of claim 4 wherein the first portion of the polynucleotide molecule is double-stranded DNA.

12. The method of claim 1 wherein the solid support is a particle.

13. The method of claim 5 wherein at least one single-stranded DNA probe which is not bound to the solid support comprises a segment which is double-stranded DNA.

14. The method of claim 4 wherein the biological sample is treated to form the single-stranded DNA analyte.

15. The method of claim 11 wherein the first portion of the polynucleotide molecule is detected using a reagent which specifically binds to double-stranded DNA.

16. The method of claim 15 wherein the reagent is a protein.

17. The method of claim 16 wherein the protein is a first antibody.

18. The method of claim 17 wherein the first antibody comprises a detectable label.

19. The method of claim 17 wherein specific binding of the first antibody to double-stranded DNA is detected by a second antibody which specifically binds to the first antibody.

20. The method of claim 17 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.3 pg/µl.

21. The method of claim 20 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 5 fg/µl.

22. The method of claim 21 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 1 fg/µl.

23. The method of claim 22 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.5 fg/µl.

24. The method of claim 23 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.1 fg/µl.

25. The method of claim 17 wherein the first antibody is a monoclonal antibody produced using spleen cells from an animal which is susceptible to autoimmune disease.

26. The method of claim 25 wherein the animal is an MRL/lpr mouse.

27. The method of claim 1 wherein the single-stranded polynucleotide probe comprises a first binding moiety and wherein the solid support comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other.

28. The method of claim 27 wherein the first or second binding moiety is biotin.

29. The method of claim 27 wherein the first or second binding moiety is avidin.

30. The method of claim 27 wherein the first or second binding moiety is streptavidin.

31. The method of claim 27 wherein the second binding moiety is an antibody.

32. The method of claim 1 wherein the single-stranded polynucleotide probe is covalently bound to the solid support.

33. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis C virus.

34. The method of claim 33 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

35. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis B virus.

36. The method of claim 35 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9.

37. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis G virus.

38. The method of claim 37 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:13.

39. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of an HIV virus.

40. The method of claim 39 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18.

41. The method of claim 1 wherein two or more distinct DNA probes are bound to the solid support.

42. The method of 42 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

43. The method of claim 42 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

44. The method of claim 1 wherein the polynucleotide molecule comprises a second portion which is single-stranded.

45. The method of claim 44 further comprising the step of:

synthesizing at least one polynucleotide strand which is complementary to all or a part of the second portion of the polynucleotide molecule to form additional double-stranded portions of the polynucleotide molecule.

46. The method of claim 45 wherein the step of synthesizing uses a non-thermostable polymerase.

47. The method of claim 46 wherein the non-thermostable polymerase is a T4 polymerase.

48. The method of claim 45 wherein the additional double-stranded portions of the polynucleotide molecule are double-stranded DNA.

49. The method of claim 48 wherein the additional double-stranded portions of the polynucleotide molecule are detected using an antibody which specifically binds to double-stranded DNA.

50. The method of claim 49 wherein the antibody comprises a detectable label.

51. The method of claim 4, further comprising the step of:

synthesizing additional copies of the single-stranded DNA analyte prior to hybridizing the single-stranded DNA analyte with the one or more single-stranded DNA probes.

52. The method of claim 51 wherein additional copies of the complement of the single-stranded DNA analyte are not synthesized.

53. The method of claim 51 wherein the step of synthesizing employs one single-stranded DNA primer.

54. The method of claim 51 wherein the step of synthesizing proceeds by linear kinetics.

55. The method of claim 51 wherein the single-stranded DNA primer is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 4, 5, 8, 10, and 12.

56. The method of claim 51 wherein the DNA probe is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

57. The method of claim 51 wherein the step of synthesizing uses a thermostable polymerase.

58. The method of claim 57 wherein the step of synthesizing uses fewer than 45 polymerization cycles.

59. The method of claim 58 wherein the step of synthesizing uses fewer than 30 polymerization cycles.

60. The method of claim 51 wherein two or more distinct single-stranded DNA probes are bound to the solid support.

61. The method of claim 60 wherein the step of synthesizing employs two or more distinct single-stranded DNA primers which are complementary to two or more distinct single-stranded DNA analytes.

62. The method of claim 51 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

63. The method of 62 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

64. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

at least one single-stranded DNA probe which comprises a first binding moiety;

a solid support which comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other; and

a monoclonal antibody which is capable of detecting double-stranded DNA.

65. The kit of claim 64 further comprising written instructions for a method comprising the step of:

detecting a DNA molecule on a solid support, wherein the DNA molecule comprises (a) a single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

66. The kit of claim 64 further comprising written instructions for a method comprising the steps of:

synthesizing additional copies of a single-stranded DNA analyte prior to hybridizing the single-stranded DNA analyte with one or more single-stranded DNA probes; and

detecting a DNA molecule on a solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

67. The kit of claim 64 wherein the solid support is a particle.

68. The kit of claim 64 which comprises two or more distinct DNA probes.

69. The kit of claim 66 further comprising a thermostable DNA polymerase.

70. The kit of claim 66 further comprising a single-stranded DNA primer for synthesizing additional copies of the single-stranded DNA analyte.

71. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

a solid support which comprises a single-stranded DNA probe; and

a monoclonal antibody which is capable of detecting double-stranded DNA.

72. The kit of claim 71 further comprising written instructions for a method comprising the step of:

detecting a DNA molecule on a solid support, wherein the DNA molecule comprises (a) a single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

73. The kit of claim 71 further comprising written instructions for a method comprising the steps of:

synthesizing additional copies of a single-stranded DNA analyte prior to hybridizing the single-stranded DNA analyte with one or more single-stranded DNA probes; and

detecting a DNA molecule on a solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

74. The kit of claim 71 wherein the solid support is a particle.

75. The kit of claim 71 wherein the solid support comprises two or more distinct DNA probes.

76. The kit of claim 73 further comprising a thermostable DNA polymerase.

77. The kit of claim 73 further comprising a single-stranded DNA primer for synthesizing additional copies of the single-stranded DNA analyte.

78. A single-stranded DNA primer which consists of a sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 5, 8, 10, and 12.

79. The method of claim 1 further comprising the step of:

quantitating the polynucleotide molecules on the solid support which comprise first portions which are double-stranded, wherein a quantity of the polynucleotide molecules is correlated with a quantity of the single-stranded polynucleotide analyte in the biological sample.

PATENT COOPERATION TREATY

LMH

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

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NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	20.03.00
Applicant's or agent's file reference 3904.10930	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/24494	16/11/1998	03/12/1997

Applicant
DIASORIN INTERNATIONAL INC. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
3904.10930		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/24494	16/11/1998	03/12/1997
International Patent Classification (IPC) or national classification and IPC		
C12Q1/68		
Applicant		
DIASORIN INTERNATIONAL INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 11 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21/06/1999	20.03.00
Name and mailing address of the international preliminary examining authority:	Authorized officer
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Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US98/24494

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-39 as originally filed

Claims, No.:

1-119 as received on 28/02/2000 with letter of 25/02/2000

Drawings, sheets:

1/3-3/3 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

see separate sheet

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	3, 20-24, 34, 38, 40, 42-43, 45-48, 51, 52, 57, 60, 61, 67, 86-90, 100, 104, 106, 108-113
	No:	Claims	1, 2, 4-19, 25-33, 35-37, 39, 41, 44, 49, 50, 53-56, 58, 59, 62-66, 68-85, 91-99, 101-103, 105, 107, 114-119
Inventive step (IS)	Yes:	Claims	34, 40, 42, 43, 45-48, 51, 52, 60, 61, 67, 100, 104, 106, 108-113
	No:	Claims	1-33, 35-39, 41, 44, 49, 50, 53-59, 62-66, 68-99, 101-103, 105, 107, 114-119
Industrial applicability (IA)	Yes:	Claims	1-119
	No:	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

1. Section I

The amended claims 1 and 62, filed with the letter of 25.02.2000 introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 19(2) PCT.

The amendments concerned are the following:

- The feature "non-exponential kinetics" includes all non-exponential kinetics. The description (page 10 lines 21-27) and claim 52 only mention **linear** kinetics.
- The feature "one single-stranded primer" includes one single-stranded DNA and RNA primers. The description (page 10 lines 28-30) and claim 51 only disclose one single-stranded **DNA** primer.

2. Section V

Reference is made to the following documents:

D1 MANTERO G et al., CLINICAL CHEMISTRY, vol. 37, no. 3, 1 March 1991, pages 422-429.

D2 WO 97 27333 A, 31 July 1997

D3 EP 0 462 353 A, 27 December 1991

D4 WO 95 30746 A, 16 November 1995

D5 WO 97 39129 A, 23 October 1997

D6 WO 97 40193 A, 30 October 1997

D7 DATABASE GCG-GENSEQ embl AC:T76940, 28 October 1997 NIAKKU: "NonA nonB non C hepatitis virus gene" XP002095422.

D1 (page 422 column 1 lines 1-25) describes the amplification of target sequences, hybridization of amplified DNA with solid-phase bound capture probes and the detection of double-stranded hybrids by double-stranded DNA specific antibodies. D1 further discloses the nucleotide sequence of HBV primer SEQ ID 8 & HBV probe SEQ ID 9 (page 422 column 2 lines 45- page 423 column 1 line 2).

D2 disclose a method for detecting a nucleotide acid sequence in a sample comprising immobilization of target sequence or amplified target sequence by capture probe and the detection of double-stranded hybrids by double-stranded DNA specific antibodies

(claims 1, 9 & 11). D2 further discloses a kit comprising a primer pair, a solid phase labelled nucleotide probe and a double helix nucleic acid specific ligand (claim 12).

D3 discloses a method for determining the presence of a target nucleotide sequence in a sample comprising the following steps:

- the target nucleotide is denatured and amplified (optional, claim 9)
- the target nucleotide has been made available in single-stranded form (claim 1)
- hybridisation to a nucleotide probe, which is complementary to the target sequence, and which is preferably immobilised on a solid substrate (claim 8)
- detection of the probe/target hybrid by an anti-nucleic-acid antibody adapted electively to distinguish double-stranded from single-stranded sequences (claim 1)

The document further discloses a kit for carrying out the above-mentioned method (claim 11).

D4 discloses the antisense nucleotide sequence of HCV primers SEQ ID 3 & 5 (page 26, SEQ ID 2 & page 27, SEQ ID 4).

D5 discloses the nucleotide sequence of HGV primer SEQ ID 12 (page 26 SEQ ID 5).

D6 (abstract) relates to a method for detection of HBV in a biological sample, In which the polynucleic acids of the sample are hybridized with a combination of at least 2 nucleotide probes, with said probes being bound to a solid support. The document further discloses the nucleotide sequence of HBV primer SEQ ID 8 and a kit for carrying out the above-mentioned method.

D7 discloses the nucleotide sequence of HGV probe SEQ ID 13 (abstract).

2.1 The subject-matter of claim 1 does not meet the requirements of Article 33(2) PCT. As a result of the unclear wording of the amplification process, the synthesizing step of claim 1 is considered to encompass a conventional PCR amplification reaction (see section VIII, point 4.1 below).
D1-D3 & D6 anticipate the novelty (Article 33(2) PCT) of the subject-matter of claim 1.

2.2 The subject-matter of claims 2, 4-19, 25-33, 35-37, 39, 41, 44, 49, 50, 53-56, 58,

59 & 68 is not novel (Article 33(2) PCT).

- D1 is prejudicial to the novelty of claims 2, 4-11, 13-18, 25-29, 31, 32, 35, 36, 39, 44, 53-56 & 68
- D2 is prejudicial to the novelty of claims 2, 4, 5, 7-11, 14, 15, 25, 27, 32, 33, 37, 44, 49, 50, 55, 56 & 68
- D3 is prejudicial to the novelty of claims 2, 4, 5, 11-19, 25-32, 35, 44, 55 & 68
- D6 anticipates the novelty of claims 2, 4, 5, 7-11, 15, 32, 35, 41, 44, 53, 58, 59 & 68.

2.3 "Binding the probe to the solid support after hybridization" (claim 3), "the sensitivity of the method of detection" (claims 20-24) and "the step of synthesizing using fewer than 30 polymerization cycles" (claim 57) are not disclosed in any of the available prior art documents and therefore novel (Article 33(2) PCT). However, these technical features are merely one of several straightforward possibilities from which the skilled person would select, in accordance with circumstances, without the exercise of inventive skill. Therefore, the subject-matter of claims 3, 20-24 & 57 is considered not to involve an inventive step (Article 33(3) PCT).

2.4 The nucleotide sequences SEQ ID NO:6 (claim 34), SEQ ID NO:13 (claim 38), SEQ ID NO:18 (claim 40) of the DNA probes render these claims novel over D3, which is considered to represent the closest prior art.
SEQ ID NO:13 (claim 38) is disclosed in D7. The subject-matter of claim 38 is therefore considered not to involve an inventive step (Article 33(3) PCT). There is no indication of the other sequences in the available prior art. Thus, the subject-matter of claims 34 & 40 is inventive (Article 33(3) PCT). Claims 42, 43, 60 & 61 are inventive for the same reasons.

2.5 The additional feature "the detection method further comprises a step of synthesizing additional copies of the double-stranded portions of the polynucleotide molecule (claims 45-48)" renders the claim novel over D3, which is considered to represent the closest prior art. There is no indication in any of the available prior art documents of an extra amplification step of the double-stranded formed hybrids. Therefore, the subject-matter of claims 45-48 is considered to

involve an inventive step (Article 33(3) PCT).

2.6 The subject-matter of claims 51 & 52 differs from D3, which is considered to represent the closest prior art, only in that:

- **use of only one single-stranded DNA primer (claim 51)**
- **the step of synthesizing proceeds by linear kinetics (claim 52)**

There is no indication in any of the available prior art documents of the above-mentioned amplification characteristics. Therefore, It is not obvious for a skilled person to perform said amplification reaction. Thus, the subject-matter of claims 51 & 52 is considered to involve an inventive step (Article 33(3) PCT).

2.7 The subject-matter of claims 62-66 is not novel (Article 33(2) PCT) for the same reason as explained in point 2.1 above.

2.8 The subject-matter of claim 67 is novel and inventive (Article 33(2) and 33(3)PCT) because none of the available prior art documents discloses or suggest single-stranded DNA primers consisting of the nucleotide sequences SEQ ID NOs:1, 2 and 10.

2.9 D1-D3 & D6 anticipate the novelty (Article 33(2) PCT) of claim 69.
For the examination for novelty and inventive step of dependent claims 70-116, reference is made to points 2.2, 2.3, 2.4, 2.5 & 2.6 above.

2.10 D1-D3 & D6 anticipate the novelty (Article 33(2) PCT) of claim 117. D3 (page 2 column 2 lines 50-58) is further prejudicial to the novelty (Article 33(2) PCT) of claim 118 and D6 to the novelty of claim 119.

3. Section VII

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D3 & D6 are not mentioned in the description, nor are these documents identified therein.

4. Section VIII

4.1 The sentence "synthesizing additional copies of the single-stranded polynucleotide analyte, wherein additional copies of the complement of the single-stranded polynucleotide analyte are not synthesized" used in claim 1 & 62 is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT). Since an amplification reaction is based on synthesizing the complement of a polynucleotide sequence on which the primer is annealing, it is not clear how one can synthesize additional copies without having the complement of the single stranded polynucleotide analyte.

4.2 The term "to all or a portions of " used in claims 33, 35, 37, 39, 42, 45, 60, 99, 101,103, 105 & 108 is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT). The particular identifying characteristics of said portions should be included in order to exclude variants which are not further specified.

4.3 The length of the DNA probe as described in the claims 8-10 & 74-76 of the present invention and the length of the DNA probe described in the description (page 5 line 30-page 6 line 2) are not the same. This inconsistency between the claims and the description leads to doubt concerning the matter for which protection is sought, thereby rendering the claims unclear (Article 6 PCT).

4.4 The number of dependent claims can be reduced by using multiple back references.

CLAIMS

1. A method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample, comprising the step of:

synthesizing additional copies of the single-stranded polynucleotide analyte, wherein additional copies of the complement of the single-stranded polynucleotide analyte are not synthesized; and

detecting a polynucleotide molecule on a solid support, wherein the polynucleotide molecule comprises (a) the single-stranded polynucleotide analyte and (b) one or more single-stranded polynucleotide probes hybridized to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded, wherein at least one of the single-stranded polynucleotide probes is bound to the solid support, wherein detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

2. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support prior to hybridization with the single-stranded polynucleotide analyte.

3. The method of claim 1 wherein the at least one single-stranded polynucleotide probe is bound to the solid support after hybridization with the single-stranded polynucleotide analyte.

4. The method of claim 1 wherein the single-stranded polynucleotide analyte is DNA.

5. The method of claim 1 wherein the one or more single-stranded polynucleotide probes are DNA probes.

6. The method of claim 5 wherein <u>at least one of</u> the <u>one or more</u> DNA [probe] <u>probes</u> is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

7. The method of claim 5 wherein one or more of the DNA probes is 20-100 nucleotides in length.

8. The method of claim 5 wherein one or more of the DNA probes is

less than 20 nucleotides in length.

9. The method of claim 5 wherein one or more of the DNA probes is less than 50 nucleotides in length.

10. The method of claim 5 wherein one or more of the DNA probes is less than 100 nucleotides in length.

11. The method of claim 5 wherein the first portion of the polynucleotide molecule is double-stranded DNA.

12. The method of claim 1 wherein the solid support is a particle.

13. The method of claim 5 wherein at least one single-stranded DNA probe which is not bound to the solid support comprises a segment which is double-stranded DNA.

14. The method of claim 4 wherein the biological sample is treated to form the single-stranded DNA analyte.

15. The method of claim 11 wherein the first portion of the polynucleotide molecule is detected using a reagent which specifically binds to double-stranded DNA.

16. The method of claim 15 wherein the reagent is a protein.

17. The method of claim 16 wherein the protein is a first antibody.

18. The method of claim 17 wherein the first antibody comprises a detectable label.

19. The method of claim 17 wherein specific binding of the first antibody to double-stranded DNA is detected by a second antibody which specifically binds to the first antibody.

20. The method of claim 17 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.3 pg/μl.

21. The method of claim 20 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 5 fg/μl.

22. The method of claim 21 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 1 fg/μl.

23. The method of claim 22 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.5 fg/μl.

24. The method of claim 23 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.1 fg/μl.

25. The method of claim 17 wherein the first antibody is a monoclonal antibody produced using spleen cells from an animal which is susceptible to autoimmune disease.

26. The method of claim 25 wherein the animal is an MRL/lpr mouse.

27. The method of claim 1 wherein the single-stranded polynucleotide probe comprises a first binding moiety and wherein the solid support comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other.

28. The method of claim 27 wherein the first or second binding moiety is biotin.

29. The method of claim 27 wherein the first or second binding moiety is avidin.

30. The method of claim 27 wherein the first or second binding moiety is streptavidin.

31. The method of claim 27 wherein the second binding moiety is an antibody.

32. The method of claim 1 wherein the single-stranded polynucleotide probe is covalently bound to the solid support.

33. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis C virus.

34. The method of claim 33 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

35. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis B virus.

36. The method of claim 35 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9.

37. The method of claim 1 wherein the single-stranded polynucleotide

probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis G virus.

38. The method of claim 37 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:13.

39. The method of claim 1 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of an HIV virus.

40. The method of claim 39 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18.

41. The method of claim 1 wherein two or more distinct DNA probes are bound to the solid support.

42. The method of 41 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

43. The method of claim 42 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

44. The method of claim 1 wherein the polynucleotide molecule comprises a second portion which is single-stranded.

45. The method of claim 44 further comprising the step of:

synthesizing at least one polynucleotide strand which is complementary to all or a part of the second portion of the polynucleotide molecule to form additional double-stranded portions of the polynucleotide molecule.

46. The method of claim 45 wherein the step of synthesizing uses a non-thermostable polymerase.

47. The method of claim 46 wherein the non-thermostable polymerase is a T4 polymerase.

48. The method of claim 45 wherein the additional double-stranded

portions of the polynucleotide molecule are double-stranded DNA.

49. The method of claim 48 wherein the additional double-stranded portions of the polynucleotide molecule are detected using an antibody which specifically binds to double-stranded DNA.

50. The method of claim 49 wherein the antibody comprises a detectable label.

51. The method of claim 1 wherein the step of synthesizing employs one single-stranded DNA primer.

52. The method of claim 1 wherein the step of synthesizing proceeds by linear kinetics.

53. The method of claim 1 wherein the single-stranded DNA primer is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 4, 5, 8, 10, and 12.

54. The method of claim 1 wherein the DNA probe is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

55. The method of claim 1 wherein the step of synthesizing uses a thermostable polymerase.

56. The method of claim 55 wherein the step of synthesizing uses fewer than 45 polymerization cycles.

57. The method of claim 56 wherein the step of synthesizing uses fewer than 30 polymerization cycles.

58. The method of claim 1 wherein two or more distinct single-stranded DNA probes are bound to the solid support.

59. The method of claim 58 wherein the step of synthesizing employs two or more distinct single-stranded DNA primers which are complementary to two or more distinct single-stranded DNA analytes.

60. The method of claim 1 wherein the solid support comprises a first DNA probe which is complementary to a portion of a polynucleotide sequence of an HIV virus, a second DNA probe which is complementary to a portion of a polynucleotide sequence of a hepatitis B virus, and a third DNA probe which is

complementary to a portion of a polynucleotide sequence of a hepatitis C virus.

61. The method of 60 wherein the first DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18, the second DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9, and the third DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

62. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

at least one single-stranded DNA probe which comprises a first binding moiety;

a solid support which comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other; [and]

a monoclonal antibody which is capable of detecting double-stranded DNA; and

written instructions for a method comprising the steps of:

synthesizing additional copies of the single-stranded DNA analyte prior to hybridizing the single-stranded DNA analyte with the at least one single-stranded DNA probe, wherein additional copies of the complement of the single-stranded polynucleotide analyte are not synthesized; and

detecting a DNA molecule on the solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) at least one of the single-stranded DNA probes which is specifically hybridized to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein the at least one single-stranded DNA probe is bound to the solid support, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

63. The kit of claim 62 wherein the solid support is a particle.

64. The kit of claim 62 which comprises two or more distinct DNA probes.

65. The kit of claim 62 further comprising a thermostable DNA polymerase.

66. The kit of claim 62 further comprising a single-stranded DNA primer for synthesizing additional copies of the single-stranded DNA analyte.

67. A single-stranded DNA primer which consists of a sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:1, 2, 3, 5, 8, 10, and 12.

68. The method of claim 1 further comprising the step of:

quantitating the polynucleotide molecules on the solid support which comprise first portions which are double-stranded, wherein a quantity of the polynucleotide molecules is correlated with a quantity of the single-stranded polynucleotide analyte in the biological sample.

69. A method of detecting the presence of a single-stranded polynucleotide analyte in a biological sample, comprising the step of:

detecting a polynucleotide molecule on a solid support, wherein the polynucleotide molecule comprises (a) the single-stranded polynucleotide analyte and (b) one or more single-stranded polynucleotide probes hybridized to the single-stranded polynucleotide analyte to form one or more first portions of the polynucleotide molecule which are double-stranded, wherein at least one of the single-stranded polynucleotide probes is bound to the solid support after hybridization with the single-stranded polynucleotide analyte, wherein detection of the first portion of the polynucleotide molecule on the solid support indicates the presence of the single-stranded polynucleotide analyte in the biological sample.

70. The method of claim 69 wherein the single-stranded polynucleotide analyte is DNA.

71. The method of claim 69 wherein the one or more single-stranded polynucleotide probes are DNA probes.

72. The method of claim 71 wherein at least one of the one or more DNA probes is selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS:6, 7, 9, 11, 13, and 18.

73. The method of claim 71 wherein one or more of the DNA probes is 20-100 nucleotides in length.

74. The method of claim 71 wherein one or more of the DNA probes is

less than 20 nucleotides in length.

75. The method of claim 71 wherein one or more of the DNA probes is less than 50 nucleotides in length.

76. The method of claim 71 wherein one or more of the DNA probes is less than 100 nucleotides in length.

77. The method of claim 69 wherein the first portion of the polynucleotide molecule is double-stranded DNA.

78. The method of claim 69 wherein the solid support is a particle.

79. The method of claim 71 wherein at least one single-stranded DNA probe which is not bound to the solid support comprises a segment which is double-stranded DNA.

80. The method of claim 69 wherein the biological sample is treated to form the single-stranded DNA analyte.

81. The method of claim 69 wherein the first portion of the polynucleotide molecule is detected using a reagent which specifically binds to double-stranded DNA.

82. The method of claim 81 wherein the reagent is a protein.

83. The method of claim 82 wherein the protein is a first antibody.

84. The method of claim 83 wherein the first antibody comprises a detectable label.

85. The method of claim 83 wherein specific binding of the first antibody to double-stranded DNA is detected by a second antibody which specifically binds to the first antibody.

86. The method of claim 69 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.3 pg/μl.

87. The method of claim 86 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 5 fg/μl.

88. The method of claim 87 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 1 fg/μl.

89. The method of claim 88 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.5 fg/μl.

90. The method of claim 89 wherein the single-stranded DNA analyte is present in the biological sample at a concentration of less than 0.1 fg/μl.

91. The method of claim 83 wherein the first antibody is a monoclonal antibody produced using spleen cells from an animal which is susceptible to autoimmune disease.

92. The method of claim 91 wherein the animal is an MRL/lpr mouse.

93. The method of claim 69 wherein the single-stranded polynucleotide probe comprises a first binding moiety and wherein the solid support comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other.

94. The method of claim 93 wherein the first or second binding moiety is biotin.

95. The method of claim 93 wherein the first or second binding moiety is avidin.

96. The method of claim 93 wherein the first or second binding moiety is streptavidin.

97. The method of claim 93 wherein the second binding moiety is an antibody.

98. The method of claim 69 wherein the single-stranded polynucleotide probe is covalently bound to the solid support.

99. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis C virus.

100. The method of claim 99 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:6.

101. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis B virus.

102. The method of claim 101 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:9.

103. The method of claim 69 wherein the single-stranded polynucleotide

probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of a hepatitis G virus.

104. The method of claim 103 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:13.

105. The method of claim 69 wherein the single-stranded polynucleotide probe is a DNA probe which is complementary to all or a portion of a polynucleotide sequence of an HIV virus.

106. The method of claim 105 wherein the DNA probe comprises the nucleotide sequence shown in SEQ ID NO:18.

107. The method of claim 69 wherein the polynucleotide molecule comprises a second portion which is single-stranded.

108. The method of claim 69 further comprising the step of:

synthesizing at least one polynucleotide strand which is complementary to all or a part of the second portion of the polynucleotide molecule to form additional double-stranded portions of the polynucleotide molecule.

109. The method of claim 69 further comprising the step of coupling additional double-stranded DNA to the polynucleotide molecule.

110. The method of 109 wherein a dendrimer is coupled to the polynucleotide molecule.

111. The method of claim 108 wherein the step of synthesizing uses a non-thermostable polymerase.

112. The method of claim 111 wherein the non-thermostable polymerase is a T4 polymerase.

113. The method of claim 108 wherein the additional double-stranded portions of the polynucleotide molecule are double-stranded DNA.

114. The method of claim 108 wherein the additional double-stranded portions of the polynucleotide molecule are detected using an antibody which specifically binds to double-stranded DNA.

115. The method of claim 114 wherein the antibody comprises a detectable label.

116. The method of claim 69 wherein two or more distinct single-stranded

DNA probes are used.

117. A kit for detecting a single-stranded DNA analyte in a biological sample, comprising:

at least one single-stranded DNA probe which comprises a first binding moiety;

a solid support which comprises a second binding moiety, wherein the first and second binding moieties specifically bind to each other; [and]

a monoclonal antibody which is capable of detecting double-stranded DNA; and

written instructions for a method comprising the step of:

detecting a DNA molecule on the solid support, wherein the DNA molecule comprises (a) the single-stranded DNA analyte and (b) one or more single-stranded DNA probes which specifically hybridize to the single-stranded DNA analyte to form one or more first portions of the DNA molecule which are double-stranded, wherein at least one of the single-stranded DNA probes is bound to the solid support after hybridization with the single-stranded polynucleotide analyte, wherein detection of the first portion of the DNA molecule on the solid support indicates the presence of the single-stranded DNA analyte in the biological sample.

118. The kit of claim 117 wherein the solid support is a particle.

119. The kit of claim 117 which comprises two or more distinct DNA probes.

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 98/24494

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 91 19812 A (BIO MERIEUX) ✓ 12 December 1991 see claims, absract and p.8	1-5,8,12 6,37,38
X	MANTERO G ET AL: "DNA ENZYME IMMUNOASSAY: GENERAL METHOD FOR DETECTING PRODUCTS OF POLYMERASE CHAIN REACTION" CLINICAL CHEMISTRY, vol. 37, no. 3, 1 March 1991, pages 422-429, XP000371646 see the whole document	1-5,7, 10,11, 15-19, 27-30, 64,71,79

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
4 March 1999	15/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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INTERNATIONAL SEARCH REPORT

Intern: al Application Nc: PCT/US 98/24494

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 27333 A (SORIN BIOMEDICA DIAGNOST SPA ;PRIMI DANIELE (IT); FIORDALISI GIANF) 31 July 1997 see whole doc esp. claims	1-5,7,9, 11, 15-18, 33,35, 37,51, 57,58, 60,64,71
X	WO 88 01302 A (SISKA DIAGNOSTICS INC) 25 February 1988 see abstract and claims	1-5,8,9
X	EP 0 462 353 A (CONBIOTEC CONSORZIO PER LE BIO) 27 December 1991 see the whole document	1-5,11, 15-18, 51,53, 64,71,79
A	FIORDALISI G ET AL: "HIGH PREVLENCE OF GB VIRUS C INFECTION IN A GROUP OF ITALIAN PATIENTS WITH HEPATITIS OF UNKNOWN ETIOLOGY" JOURNAL OF INFECTIOUS DISEASES, vol. 174, no. 1, July 1996, pages 181-183, XP000647922 see the whole document	1-79
X	DATABASE GCG-GENSEQ embl t01509, 12 October 1995 MATSUMOTO A. ET AL.,: "Determination of nucleic copy number by comp. PCR" XP002095421 see the whole document	78
X	WO 95 35390 A (SINAI SCHOOL MEDICINE ;ZHANG DAVID Y (US)) 28 December 1995 see the whole document	78
X	WO 95 30746 A (GEN HOSPITAL CORP ;WAKITA TAKAJI (US); WANDS JACK R (US)) 16 November 1995 see the whole document	78
X	WO 97 39129 A (FIORDALISI GIANFRANCO ;PRIMI DANIELE (IT); MATTIOLI SONIA (IT); WA) 23 October 1997 see the whole document	78
X	WO 97 40193 A (ROSSAU RUDI ;INNOGENETICS NV (BE); MAERTENS GEERT (BE); STUYVER LI) 30 October 1997 see the whole document	78

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INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 98/24494

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE GCG-GENSEQ embl AC:T76940, 28 October 1997 NIAKKU: "NonA nonB non C hepatitis virus gene" XP002095422 see abstract ----	6,37,38

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Internat Application No
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WO 9740193 A	30-10-1997	AU	2766297 A	12-11-1997

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/24494

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 91 19812 A (BIO MERIEUX) ~~apparently~~ separate capture + detection probes 12 December 1991 see claims, absract and p.8 Combo w/ Nagafuchi	1-5,8,12 6,37,38
X	MANTERO G ET AL: "DNA ENZYME IMMUNOASSAY: GENERAL METHOD FOR DETECTING PRODUCTS OF POLYMERASE CHAIN REACTION" CLINICAL CHEMISTRY, vol. 37, no. 3, 1 March 1991, pages 422-429, XP000371646 see the whole document DELA presaturate avidin-coated support w/ biot. probe hybridize probe w/ denatured amplified DNA incubate w/ anti-ds DNA Ab detect	1-5,7, 10,11, 15-19, 27-30, 64,71,79

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/24494

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 27333 A (SORIN BIOMEDICA DIAGNOST SPA ;PRIMI DANIELE (IT); FIORDALISI GIANF) 31 July 1997 only DETA amplified w/ 2 primers see whole doc esp. claims	1-5,7,9, 11, 15-18, 33,35, 37,51, 57,58, 60,64,71 D2
X	WO 88 01302 A (SISKA DIAGNOSTICS INC) 25 February 1988 apparently 2 probes see abstract and claims	1-5,8,9
X	EP 0 462 353 A (CONBIOTEC CONSORZIO PER LE BIO) 27 December 1991 see the whole document	1-5,11, 15-18, 51,53, 64,71,79 D3
A general state of the art	FIORDALISI G ET AL: "HIGH PREVLENCE OF GB VIRUS C INFECTION IN A GROUP OF ITALIAN PATIENTS WITH HEPATITIS OF UNKNOWN ETIOLOGY" JOURNAL OF INFECTIOUS DISEASES, vol. 174, no. 1, July 1996, pages 181-183, XP000647922 see the whole document	1-79
X	DATABASE GCG-GENSEQ embl t01509, 12 October 1995 MATSUMOTO A. ET AL.,: "Determination of nucleic copy number by comp. PCR" ? XP002095421 see the whole document	78
X	WO 95 35390 A (SINAI SCHOOL MEDICINE ;ZHANG DAVID Y (US)) 28 December 1995 see the whole document	78
X	WO 95 30746 A (GEN HOSPITAL CORP ;WAKITA TAKAJI (US); WANDS JACK R (US)) 16 November 1995 see the whole document	78 D4
X	WO 97 39129 A (FIORDALISI GIANFRANCO ;PRIMI DANIELE (IT); MATTIOLI SONIA (IT); WA) 23 October 1997 see the whole document	78 D5
X	WO 97 40193 A (ROSSAU RUDI ;INNOGENETICS NV (BE); MAERTENS GEERT (BE); STUYVER LI) 30 October 1997 see the whole document	78 D6

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/24494

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y no win. stop	DATABASE GCG-GENSEQ embl AC:T76940, 28 October 1997 NIAKKU: "NonA nonB non C hepatitis virus gene" XP002095422 see abstract ----	6,37,38

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